

Sleep alterations in the course of aging

Environmental Inputs

Maria Panagiotou

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Sleep alterations in the course of aging
Environmental Inputs

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Do the dreams grow old?
No they don't
people grow old
and when dreams realize it they leave
in order to find new people
curl up and maybe succeed
to come true this time

Fani Athanasiadou
Translation: Georgia Matraka

To my mom, dad, Harris and in the memory of my beloved Poupi

To my Kostas, the travel buddy in my life

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Chapter 1

General introduction and aim of the project

1.1 Sleep function and mechanisms, sleep homeostasis

1.1.1 Sleep function

In Greek mythology, *Hypnos* ("sleep") was considered to be a god or a demon that comprised the personification of sleep. According to Hesiod, Hypnos had a twin brother named *Thanatos* ("death"), both being sons of *Nyx* ("The Night") and *Erebus* ("The Darkness"). Hypnos was thought to live in a cave, at the entrance of which grow a number of poppies and other hypnotic plants, where the river *Lethe* ("Forgetfulness") comes from and where night and day meet. No light and no sound would ever enter his grotto [1].

Aristotle argued that sleep arises from the concentrated hot matter (moist and solid) exhaled from ingested food, and that in the course of sleep, the organ of sense perception should lose its power after its use, so that no animal perpetually actualizes its powers. From Aristotle's definition, our understanding of sleep is much evolved, however, it is still not fully conceived. Sleep resembles a death or coma-like state, but differs from it as it is easily reversible. Until the 1950's, sleep was considered a passive, dormant part of daily life, during which brain activity was almost absent [2]. Scientists such as Pavlov and Sherrington abided by this viewpoint [3, 4]. These assumptions were debunked by the discovery of regular cyclic alterations of rapid eye movement and non-rapid eye movement sleep phases, which was a springboard to the belief that sleep is actually an actively regulated process [2, 5]. Following decades of scientific work, sleep can be defined now as an easily reversed state of reduced responsiveness, associated with a particular immobile posture and a specific sleeping site, during which the brain is highly active [6].
Almost all animals on the planet sleep, nevertheless, whether sleep is essential remains an enigma [7]. Several theories have been proposed in the attempt to elucidate the function of sleep [8, 9, 10]. *First*, it has been proposed that sleep serves energy conservation [11, 12]. Rather like recharging a battery, it is proposed in this theory that during sleep, energy resources can be conserved for a considerable amount of time, so that survival is ensured when food is scarce. Energy savings may have been involved in the sleep process and selection during evolution. The findings that sleep cycle length and sleep amounts are negatively correlated with body and brain size, with smaller animals having higher metabolic rates, smaller body size and longer sleep, further support this theory [11, 13].
Second, sleep has been thought to serve learning and memory consolidation, and performance restoring [8, 14]. This theory proposes that, while memory encoding and retrieval takes place during waking, sleep promotes memory consolidation. During sleep, newly encoded memories are transformed into more stable representations and integrate into the network of pre-existing long-term memories [15, 16]. In addition, an interesting role of sleep regarding performance restoration has been suggested, since studies showed that performance, which was impaired after sleep deprivation, could be enhanced by sleep [17, 18].
Third, sleep has been proposed to serve a glymphatic function [19]. The 'glymphatic system', being termed as such due to its dependence upon glial cells and its performance of peripheral 'lymphatic' functions in the central nervous system, has been described as a biomolecule clearance system that uses convective flow between the cerebrospinal

fluid and interstitial fluid, so that toxic metabolites in the brain are removed, during sleep [19, 20, 21]. Despite the fact that the 'glymphatic system' concept is relatively novel, studies show that failure of glymphatic function is associated with pathology in neurodegenerative disorders, traumatic brain injury as well as stroke occurrence [19, 20, 21].
Fourth and last theory poses that sleep serves a neuronal plasticity function [8, 22, 23]. In this theory, it is proposed that plastic processes which occur during wakefulness lead to a net increase in synaptic strength in many brain circuits, and sleep serves as a mediator to downscale synaptic strength to a baseline level, forming the so-called synaptic homeostasis hypothesis [22, 24].
All aforementioned theories of sleep function have been studied extensively by multiple research groups leading to controversial outcomes, since pros and cons on each theory have been expressed. We spend one third of our lives asleep, and despite the poetically romantic view of Shakespeare on sleep, that we only sleep perchance to dream, although not fully comprehended why, we undeniably need sleep.

1.1.2 Measuring Sleep: the electroencephalogram (EEG)

In the beginnings of the previous century, Hans Berger was the first to publish a series of scientific articles on the remarkable electric effect which he detected in human subjects by recording from electrodes applied to the head [25, 26]. He noted a rhythmic oscillation of potential with a frequency around 10/second, appearing when the subject lies quietly with eyes closed and disappearing if the attention was fully occupied. Berger recorded it by pad electrodes on the scalp or by needles that reached the periosteum of the skull, which he named electroencephalogram (EEG). Since this oscillation was the first one being distinguished from the EEG, it was named the alpha rhythm. Berger concluded that the potential changes were produced by the cerebral cortex and that they represent a fundamental activity of the brain. Although different from humans, potential changes were also found in the exposed cortex of animals [27]. Around the same time, the EEG was used to investigate sleep recordings in humans and animals, mainly in dogs and cats [28, 29, 30].
The EEG signal is the commonly used signal for sleep stages classification. Together with the electromyogram (EMG) for muscle activity and the electrooculogram (EOG) for eye movements recordings, they are excellent biomarkers of sleep/wake states [31]. The EEG and EMG are the recordings of the electrical field activity of large groups of cortical neurons and muscle cells, respectively [32]. The first standard method of sleep recording and manually sleep staging in humans is based on the criteria proposed by Rechtschaffen and Kales [33]. According to them, sleep consists of six different vigilance stages, namely the wakefulness, the non-rapid eye movement (NREM) sleep stage 1 (S1) which is characterized by active muscle tone and slow eyes rolling movements, the NREM sleep stage 2 (S2) in which sleep spindles and K-complexes appear, NREM sleep stage 3 (S3) and NREM sleep stage 4 (S4) in which the responsiveness to external stimuli is really decreased and rapid eye movement sleep (REM) characterized by rapid eye movements and skeletal muscles atonia. During wakefulness, the EEG shows low-voltage fast activity and high muscle tone. As NREM sleep becomes deeper, there is slowing of EEG frequencies and increasing synchronization of cortical neuronal activity, while at its deepest point the neuronal synchrony appears as large slow wave activity on

the EEG [34]. In contrast to NREM sleep which shows high amplitude low-frequency EEG activity, reduced muscle tone and slow rolling eye movements, REM sleep is characterized by low-voltage fast activity, however the muscle tone is entirely diminished (REM muscle atonia), there is cortical desynchrony and the typical rapid eye movements can be distinguished, being associated with dream states [32, 34]. The newer method of sleep staging, developed by the American Academy of Sleep Medicine (AASM), combines sleep stages S3 and S4 of into a single stage, known as slow-wave sleep (SWS) or deep sleep [35]. In humans, healthy night time sleep consists of four to five 90-min cycles of NREM and REM sleep per night with NREM sleep being more prevalent in the beginning and REM sleep more prevalent toward the end of the night. In mammals and other animals, sleep stages may differ, since less elaborate characteristics compared to humans are evident. In rodents, mainly three stages are scored, namely waking, NREM sleep and REM sleep. The parameters extracted from the EEG signal can be used for analysis and classification of the stages of sleep. Specifically, power spectral features, based on Fast Fourier transform (FFT) of the EEG signal, have been used for classification and analysis of sleep stages [36]. Figure 1.1 shows an example of Waking, NREM sleep and REM sleep EEG and EMG recording signals from a mouse after electrode implantation 1.1.
In rodents, in contrast to human polysomnography recordings where EOG is commonly used, mostly EEG and EMG signals are used. Waking EEG, as shown in Figure 1.1, is characterized by the presence of activity between 6-9 Hz, which is thought to arise by a physical spread of theta activity from the hippocampus [37, 38, 39]. Hippocampal theta activity has been associated with voluntary activity, arousal, attention, the representation of spatial position as well as learning. Analogous EEG frequencies are found to characterize REM sleep, also being associated with hippocampal functioning [40, 41]. During NREM sleep, neurons in the cortex and thalamus are bistable, oscillating every second, formulating the distinctive feature of the EEG which is the near-synchronous occurrence of slow waves in the cortical areas prevailing between 0.5-4.0 Hz [42, 43]. Underlying the sleep slow waves, a fundamental cellular phenomenon exists, namely the slow oscillation. The slow oscillation, being the result of disfacilitation (i.e. a lack of synaptic input) consists of an up state, which is characterized by sustained neuronal depolarization and irregular firing, and it is followed by a hyperpolarized down state, during which the cortical cell ceases firing [44, 45, 46, 47]. Due to the fact that the slow oscillations occur more or less synchronously in many neurons, their summed activity is the one recorded from the scalp as slow waves, with the down state of thalamocortical neurons corresponding to the negative phase of the slow waves recorded from the scalp.

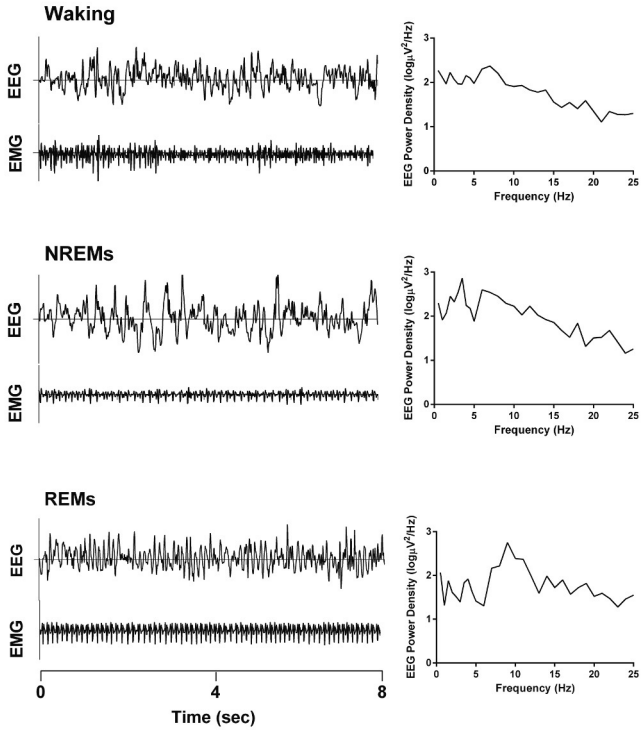

Figure 1.1: An example of raw data of Waking, NREM and REM sleep electroencephalogram (EEG) and electromyogram (EMG) recording signals from a young mouse after electrode implantation.

1.1.3 Sleep and Brain areas

Despite the exponential growth rate of scientific progress, sleep remains one of the most puzzling, yet ubiquitous behaviors across the animal kingdom. One hundred years ago, eminent scientists doubted even the existence of specific neural pathways for wakefulness and sleep regulation. Subsequent research has, however, altered dramatically this picture, and today the neural mechanisms underlying the sleep-wake states have been delineated and are portrayed in the next paragraphs.

Sleep and wakefulness are controlled by a balance between sleep-promoting and wake-promoting nuclei [48]. The ascending arousal system, originating in the brainstem, can arouse the cerebral cortex to promote wakefulness either directly or indirectly via two major branches (Figure 1.2, left panel: A, wake-promoting pathways) 1.2. One branch primarily originates from the pedunculopontine and laterodorsal tegmental nuclei (PPT and LDT) that produce acetylcholine [49], ascending to and activating the relay neurons and the reticular nucleus of the thalamus. Neurons in the lateral hypothalamic area and the basal forebrain are activated by the second branch of the ascending arousal system [50, 51, 52]. Monoaminergic neurons in the upper brainstem and caudal hypothalamus,

including the noradrenergic locus coeruleus (LC), serotoninergic dorsal (DR) and median raphe nuclei, dopaminergic ventral periaqueductal grey matter and histaminergic tuberomammillary neurons, mark the beginning of this pathway.
Markedly, during sleep, the main components of the ascending arousal system become inhibited by projections originating from the ventrolateral preoptic nucleus (VLPO) (Figure 1.2, left panel: B, sleep-promoting pathways) 1.2 [53, 54, 55, 56]. The VLPO can, also, be inhibited by the afferents coming from the monoaminergic arousal systems (Figure 1.2, left panel: C) [57]. A mutual inhibition hence exists, creating this way a flip-flop switch (Figure 1.2, right panel) 1.2. Due to the sharp wake-sleep transitions it allows, the flip-flop switch is rather unstable. Therefore, specific neurons in the lateral hypothalamus, releasing orexin (also called hypocretin) serve to stabilize this switch, via their strong excitatory actions on wake-promoting neurons without disrupting sleep. Finally, REM sleep is generated by neurons around the LC region and sublaterodorsal region in the upper pons. The latter area projects to the basal forebrain and cortex, leading to dream states, as well as to medullary and spinal cord regions in order to inhibit movements during REM sleep [58].

1.1.4 Sleep homeostasis

The concept of a *milieu intérieur*, i.e. internal environment, in physiological processes was first developed in the 19th century, a notion that was later formulated as *homeostasis* [60, 61]. Elaborating, homeostasis, generally, refers to any self-regulated biological process by which organisms tend to maintain internal stability, while adjusting to conditions optimal to survival. The homeostasis term has also been applied to sleep, denoting a basic principle of sleep regulation. Particularly, sleep homeostasis has been described as an internal timer or counter which generates a sleep drive (i.e. pressure to sleep) as a function of the amount of time elapsed since the last adequate sleep episode [62]. In other words, prolonged periods of wake are followed by long periods of deep NREM sleep. This homeostatic response is mediated by NREM sleep-promoting substances, including adenosine, prostaglandin D2, and cytokines such as interleukin-1 and tumor necrosis factor-a [63]. Recent studies have focused on the neuromodulator adenosine, since it is the best understood of these somnogens. A link has been proposed between adenosine, energy metabolism, neuronal activity and sleep [64, 65, 66]. More specifically, adenosine promotes sleep, through A1 and A2a receptor agonists, by directly inhibiting wake-promoting neurons and by activating sleep-promoting neurons. As a result, stimulants such as caffeine and theophylline, serving as antagonists at both A1 and A2A adenosine receptors, can counteract the sleep-inducing effects of adenosine via neurons in the nucleus accumbens possibly projecting to wake-promoting brain regions [67, 68].

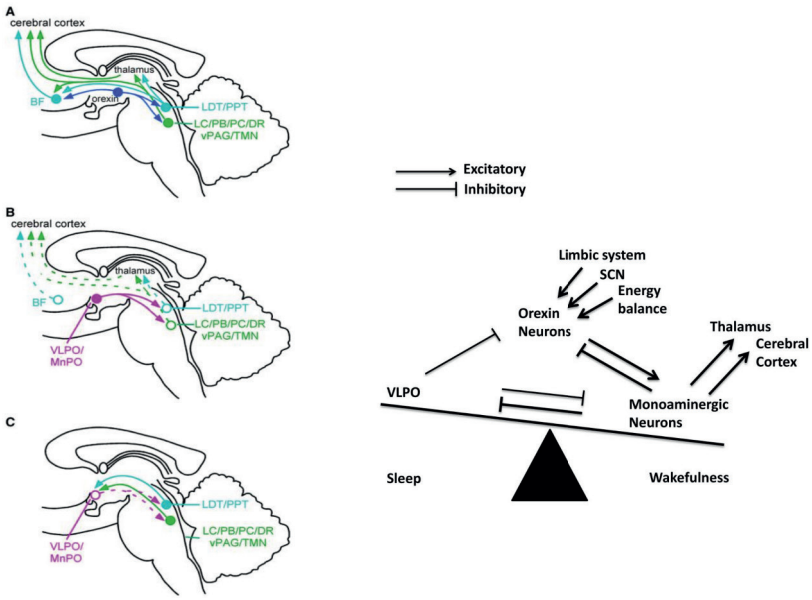

Figure 1.2: *Left panels,* Brain circuits in sleep and wakefulness (*details in the text*). **A.** Wake-promoting projections: Cholinergic neurons (light blue) comprise the major input to the thalamus, while monoaminergic and glutamatergic neurons (green) provide direct innervation of the hypothalamus, BF and cerebral cortex. The orexin neurons (dark blue) reinforce activity in these pathways as well as excite the cerebral cortex and BF. **B.** Sleep-promoting pathways: The sleep-promoting pathways from the VLPO and MnPO (magenta) inhibit the components of the ascending arousal pathways. **C:** The ascending arousal systems are also capable of inhibiting the VLPO. *DR, dorsal raphe nucleus (serotonin); LC, locus coeruleus (norepinephrine); LDT, laterodorsal tegmental nucleus (acetylcholine); PB, parabrachial nucleus (glutamate); PC, precoeruleus area (glutamate); PPT, pedunculopontine tegmental nucleus (acetylcholine); TMN, tuberomammillary nucleus (histamine); vPAG, ventral periaqueductal gray (dopamine).* (*Adapted from Saper et al., 2010* [48])
Right panel, The flip-flop switch during the state of wakefulness: Orexin neurons send excitatory input to monoaminergic neurons, which send inhibitory feedback projection to orexin neurons. This system maintains the activity of monoaminergic neurons. Monoaminergic neurons send inhibitory projection to the VLPO sleep center and send excitatory projections to the thalamus and cerebral cortex. Orexin neurons receive input from the limbic system and also have reciprocal links with the hypothalamic arcuate nucleus that regulates feeding. Additionally, orexin neurons have important role as a link between the energy homeostasis and vigilance states. *VLPO, ventrolateral preoptic nucleus; SCN, suprachiasmatic nucleus.* (*Adapted from Natsuko Tsujino & Takeshi Sakurai, 2009* [59])

Due to the rotation of the earth every 24 hours, living organisms evolved to adopt a *circadian rhythm* which is compatible with the light-dark cycle for their protection, feeding, mating and survival [69]. In humans, the intrinsic period of the circadian rhythm

is slightly longer than 24 h, whereas in mice it is about 23.5 h [70] and, hence, the circadian system comprises a mechanism which synchronizes the internal state of the organism with the predictable environmental changes. Nearly all peripheral tissues in the body have a circadian rhythm which is regulated and synchronized by a central circadian pacemaker, that in mammals is located in the suprachiasmatic nucleus (SCN) of the hypothalamus [71]. The master clock is entrained daily to 24 h by environmental cues, such as light as well as the feeding and activity patterns of the organism [72]. More specifically, the 24-h rhythms are generated by individual SCN neurons and are controlled by a molecular feedback loop [73]. It is important that the activity of these single-cell oscillators is coupled, in order to achieve adequate amplitude to either induce or suppress behavioral characteristics for specific phases of the cycle [69]. Among many physiological processes occurring in the body, the circadian clock controls also the sleep/wake cycle [71]. Unlike the aforementioned homeostatic control of sleep, circadian-controlled processes are driven by an autonomously rhythmic system. It has been proposed that the SCN is possibly unique in the entire mammalian brain since its neurons constitute a coupled, intercellular network of self-sustained, circadian oscillations of both neuronal activity and gene expression, even in the absence of external stimuli [74, 75]. Circadian rhythm outputs can be measured in many ways, for example the rest-activity rhythm can be measured using actigraphy over several days [76].

The timing, depth and duration of sleep are considered to be regulated by the interaction of these two processes, the sleep homeostatic and circadian process (*Process S and C*, respectively) as proposed by the so-called two-process model of Borbely [77] (Figure 1.3) 1.3. Process S, representing sleep debt, increases during wakefulness, declines during sleep, and oscillates with a periodicity normally entrained to day and night by the circadian pacemaker. When S is at the lower boundary, it triggers awakening whereas near the upper boundary it triggers sleep.

In mammals, the homeostatic sleep process is thought to be reflected in the NREM sleep electroencephalographic (EEG) slow-wave activity (SWA), which is the EEG power density between 0.5-4.0 Hz. EEG SWA increases in the course of waking, having its maximum level at the beginning of the sleep period, and declines during sleep showing its lowest values just before awakening [78, 79]. EEG SWA consists of two rhythms that have different brain origin and dissimilar neuronal mechanisms [80, 81]. The first one is the slow neocortical rhythm (<1Hz) which is generated within the cortex [80]. Through high-density EEG recordings, it has been shown that each cycle of the slow oscillation reflects a traveling wave which originates in prefrontal-orbitofrontal sites and propagates towards more posterior cortical areas [42]. The important role of the cortical slow oscillation is to synchronize the activity of cortical and thalamic neurons that generate spindle and delta waves throughout NREM sleep [82]. The second rhythm refers to a faster oscillation in the slow frequencies (1-4 Hz) generated in the thalamus, specifically by the interplay of two intrinsic currents of thalamocortical neurons [47]. SWA being the combination of these two rhythms is widely considered to be a measure of sleep need and/or intensity. Therefore, in the event of prolonged wakefulness, SWA increases in the subsequent sleep period in both humans and animals [78, 79]. The dynamics of sleep regulation have been extensively studied in humans and animals with the use of mathematical modeling of the observed homeostatic sleep response, visible in the EEG SWA, before and after sleep deprivation [83, 84, 85, 86]. In the next section, the effects and

applications of sleep deprivation are discussed.

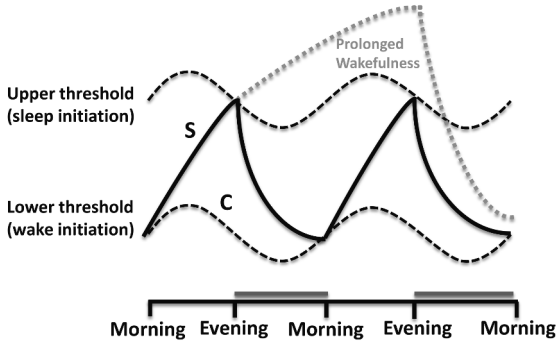

Figure 1.3: The sleep-wake regulation: the interaction between sleep homeostatic (Process S) and circadian process (Process C). Prolonged wakefulness increases sleep pressure, leading to increased total sleep time and slow-wave-activity in NREM sleep (SWA) in recovery sleep. (*Adapted from Borbely et al., 2016* [78].)

1.1.5 Sleep deprivation

In humans, the physiological sleep drive can be overruled by voluntary sleep restriction, or it can be disrupted owing to environmental factors and/or sleep disorders, for example obstructive sleep apnea, insomnia, restless leg syndrome, narcolepsy [87]. In general, sleep disruption or deprivation is detrimental for health and well-being [88]. Numerous findings in the last decades support this notion. Notably, even one night of sleep deprivation, increases cortisol levels and potentiates the HPA axis [89]. Night shift work, a common work schedule in a variety of professions, is associated with short sleep of less than 5 h [90, 91]. Sleep disruption or deprivation has been shown to impact metabolism and the immune system [87, 92, 93, 94, 95], with short sleep being associated with an increased prevalence of type 2 diabetes, obesity, and cardiovascular disease [96, 97, 98, 99]. Furthermore, cognitive performance is compromised following acute and chronic total sleep deprivation but also chronic sleep restriction, showing that sleep debt has a neurobiological cost which accumulates over time [100]. In animal studies, it has been demonstrated that sleep fragmentation impairs memory consolidation [101] and is a risk for Alzheimer's disease [102, 103]. In the most extreme case, continuous sleep deprivation can even kill rodents and flies within a period of days to weeks [104]. In order to test the sleep homeostatic process and investigate sleep characteristics under elevated sleep pressure conditions, sleep deprivation has been experimentally used in the laboratory, as some of the earlier studies, reported in this section, showed. The increased sleep pressure, as an effect of sleep deprivation, leads to alterations in the sleep-wake regulation which consist of increased total sleep time, accompanied by EEG changes

(Figure 1.3) 1.3. The sleep rebound, i.e. the sleep recovery following sleep loss, is characterized by increased sleep duration and intensity. A possible reduction in the recovery sleep, which is typically quantified as the percentage of sleep lost that is recovered over a defined time period, could underlie an impaired homeostat, unable to provide sufficient sleep or a highly efficient homeostat that is capable of restorative effects with very little sleep [105]. As aforementioned, SWA in NREM sleep is a well established electrophysiological marker of sleep homeostasis in mammals and a quantitative measure of the amplitude and incidence (number per minute of NREM sleep) of sleep slow waves [106] and it is found to be increased following sleep deprivation.

1.2 Aging: theories, sleep and the brain

1.2.1 Aging theories

Ambrosia and *Nectar*, nutriments once thought to grant immortality, are not to be found on Earth for human beings. Antithetically, aging and mortality signify an endpoint that almost all organisms on the planet experience. Potential absence of aging composes the ideal concept of biological immortality. Although this is utopian for human beings, it is actually incarnated in simple organisms such as the *Hydra* genus, which does not appear to die of old age, or to age at all, having a regenerative ability [107]. The question therefore arises, is it the complexity of higher-order creatures that enables them to senesce?
Philosophers, from the beginning of cultures, have dealt with the deeper idea and understanding of aging. A writer and philosopher of modern times said: *"In the temporary living beings, two streams struggle, first the upslope towards synthesis, life and immortality, and second, the downslope towards decomposition, matter and death" (Nikos Kazantzakis) [108].* From a scientific point of view, attempts have been made in order to elucidate the nature of aging and more importantly, whether aging can be reversed. Generally, aging can be defined as the condition where all physiological mechanisms decline, rendering an organism closer to its "death".
Aging comprises a markedly complicated and multifactorial process, thus numerous theories have been proposed over the years [109]. These can be classified into several groups of theories that may overlap at various levels of organization, being sometimes complementary with each other.
One group of theories, known as the "error theories", generally identifies several environmental insults to organisms, inducing progressive damage at various levels, e.g. mitochondrial DNA damage, oxygen radicals accumulation, cross-linking [109, 110]. The "general error theory" suggests a decline in the fidelity of gene expression with aging, leading to increased fraction of abnormal proteins [111, 112]. This erroneous synthesis can in turn generate a positive feedback of errors ultimately leading to a lethal 'error catastrophe'; however, despite the presence of error, there may be some stability of translation achieved [111, 112, 113].
Another theory which can also be categorized in the "error theories" group and has received a lot of attention, is the "free radical theory". First proposed in 1950's, it suggests that, the release of active free radicals eventually results in cumulative damage and senescence [114]. These free radicals are naturally produced by the organisms during energy production, in which mitochondrial respiration generates reactive oxygen species (ROS)

by leaking intermediates from the electron transport chain, rendering the internal environment of all living organisms into a container of free ROS [115]. Interestingly, elevated levels of oxidant-damaged DNA as well as protein have been found in aged organisms [116, 117].
The main rival theory to the "error theories" poses that aging is genetically programmed in a similar way as morphogenesis. According to these so-called "programmed theories", aging depends on biological "clocks" that regulate the time of the life span based on genes that sequentially switch on and off signals to the nervous, endocrine, and immune systems being in turn responsible for maintenance of homeostasis and for activation of defense responses [109, 110].
Furthermore, "evolutionary theories" have been formulated, in which it is supported that the deterioration observed in the course of aging results from a decline in the force of natural selection [110, 118]. In contrast to the previously discussed theories, in the evolutionary theories, aging is not programmed but an accumulation of somatic damage occurs due to limited investments in maintenance and repair beyond the age of reproduction [119].
In order to understand the insights of the aging process especially for organisms such as mammals, being extremely complex, the overall interactions among intrinsic (genetic), extrinsic (environmental), and stochastic (random damage to vital molecules) causes should be studied in a more holistic approach [110].

1.2.2 The Aging Brain and Sleep

In the course of aging, brain alterations are evident in multiple levels, from molecules to morphology and brain size. Total brain volume is shown to decline with age [120], at a rate of around 5% per decade after the age of 40 [121]. Advanced age has also been associated with widespread thinning of the cerebral cortex [122]. With respect to specifically the neocortex and hippocampus, the last decades, the idea that age-related decline in neuron number through neuron death is involved in normal aging, has become less popular [123]. The prefrontal cortex of the brain has been demonstrated to be mostly affected in healthy aging, however, in both rodents and humans, changes have been reported in dendritic arbor, spines, and synapse morphology that could impact the function of hippocampal circuits, rendering also the hippocampus another greatly affected area [123, 124, 125, 126, 127]. With regards to the synapse, loss of synaptic function and number is probably also a contributing factor in age-related cognitive decline [128, 129].
In addition, disruption of particularly myelinated fibers that connect neurons in different cortical regions occurs in the aging brain, suggesting a systems-level breakdown of integrated function that correlates with poor cognitive performance [130]. Aging has also been shown to affect white matter density, with great reductions found in the prefrontal cortex and anterior corpus callosum [131, 132].
Neurotrophic factors likely play an important role in the aging brain, since increased concentrations of them can restore cognitive functions in aged animals [133]. For example, the expression of brain-derived neurotrophic factor in the hippocampus has been found to decrease with age, an effect that may contribute to age-related cognitive impairments [134]. In addition to neurotrophic factors, neurotransmitters playing naturally an impor-

tant role in the brain, show alterations in the course of aging, that could ultimately lead to neurodegenerative diseases. Recent research has identified serotonin as a prominent signal, acting in concert with brain-derived neurotrophic factor, in order to regulate aspects of neural plasticity in several brain regions. It has been demonstrated that both could become compromised in aging and age-related neurodegenerative disorders [135]. Furthermore, human postmortem and animal experimental results suggest an age-associated disturbance of the cerebral dopaminergic neuronal system [136, 137, 138, 139, 140]. Particularly, dopaminergic pathways between the frontal cortex and the striatum, levels of dopamine may decline as a function of age, and/or synapses/receptors or binding to receptors may be reduced [141]. In general, cholinergic and monoaminergic systems projecting from the basal forebrain and brainstem have been demonstrated to be functionally impaired in the course of aging, with the metabolites of achetylcholine, dopamine and noradrenaline being generally depleted in the cerebral cortex of aged rats and monkeys [142, 143].

In the aged brain incomplete or impaired functions can be detected. Stemming from interrupted pathways and brain atrophy, sleep in aging is associated with a disrupted physiology [144]. Particularly, sleep quality is considered to be progressively decreased in the course of aging in humans, owing to reduced capacity to initiate and/or maintain sleep. The elderly sleep less, reporting more often sleep complaints, and their sleep is accompanied by frequent awakenings and superficial stage 1 sleep [145, 146, 147]. The sleep EEG is characterized by lower slow-wave activity in NREM sleep, reflecting a less deep sleep, as well as reduced sigma activity (around 13 Hz) during NREM sleep, beginning at middle age [148, 149, 150].

The brain mechanisms contributing to age-related sleep alterations are not fully understood to date. A preferential impairment of the prefrontal cortex evident from neuropsychology and brain imaging techniques has been noted with age, that may intervene with the sleep process [144]. The medial prefrontal cortex expresses gray matter reductions in elderly subjects, and comprises an area where NREM sleep slow waves show a marked preponderance in origin and density over EEG derivations [144, 151]. Compared to young adults, older subjects show lower slow-wave amplitude and density, especially in prefrontal/frontal brain areas [42]. In addition, serotonin, being implicated in a variety of neural functions including sleep regulation [152], may contribute to age-related sleep alterations, since diminished populations of serotonin receptors with age have been demonstrated in rat and human brains [153]. Interestingly, changes in the suprachiasmatic nucleus in the hypothalamus, as well as other parts of the circadian timing system are considered to underlie sleep disturbances in elderly people [154, 155]. In particular, the aging SCN is characterized by decreased neuronal activity [156, 157], showing an attenuated circadian amplitude, due to changes in neuronal phase distribution [158]. Finally, vasopressin, one of the main peptides in the SCN, declines in amount and synthesis in aged humans, leading eventually to disturbed sleep-wake rhythms [159].

Concluding, aging is a rather slow process that potentially impacts all the organs including the brain. Along with many physiological processes, sleep is consequently largely affected, rendering the elderly vastly vulnerable, as sleep remains a vital physiological process.

1.3 Environmental factors, health and aging

The so-wanted elixir of youth has not been discovered yet. However, many factors may intervene in the aging process, leading to detrimental or beneficial effects, which in turn could accelerate or decelerate parts or the whole aging procedure. Aging is a physiological process that occurs asynchronously in different areas of the brain and the rate of that process is dependent on the lifestyle of the individuals. In the following paragraphs, some of the environmental factors that humans are daily exposed to are discussed.
First, diet constitutes a significant factor, directly affecting general body and brain health. In addition to health, longevity per se is believed to be promoted through diet [160, 161, 162]. Dietary interventions, such as caloric restriction and intermittent fasting can improve health during aging, reduce the risk of developing diabetes, cancer, and cardiovascular diseases and even delay brain atrophy evident in aging [163, 164, 165, 166]. A disorder commonly viewed across all ages is obesity. Obesity can be accompanied by damage to several target organs, as for example the heart, skeletal muscle, pancreas, liver, and kidney [167]. Additionally, in human obesity, structural brain changes have been identified in several brain areas [168], rendering obesity a risk factor for neurodegenerative disorders, such as Alzheimer disease [169]. Therefore, in congruence with the notion that the choice of our food defines us, diet reflects life quality.
Second, since the 1900's artificial light was introduced as another important factor that influences our daily lives. Although artificial light has ameliorated human life in a multitude of sectors, augmented exposure, particularly during times that darkness should prevail, has been associated with drawbacks in health [170]. General disruption of normal circadian time can alter the overall physiological state. Hormone balance, immune, endocrine, as well as several metabolic system disturbances may be provoked owing to light exposure at night. Shift-work, a widely-accepted type of work in modern society, can induce detrimental effects in carbohydrate and lipid metabolism, insulin resistance, hypertension, coronary heart disease, and myocardial infarction [171], being linked also with increased risk for breast cancer [172, 173, 174, 175]. Hence, artificial light exposure, specifically at night, impacts physiological processes, that may affect consequently body and brain health.
Third, physical activity constitutes one factor, that exhibits large attention regarding general health. The roots of a mind-body connection are traced back to ancient civilizations. Plato said, "*In order for man to succeed in life, God provided him with two means, musical education and physical activity. Not separately, one for the soul and the other for the body, but for the two together. With these two means, man can attain perfection" [176].* Physical activity, such as aerobic exercise, are implicated in cognition, memory and mental health improvement, as well as in promoting structural and functional plasticity in the brain [177, 178, 179, 180]. The elderly can essentially benefit by exercise, since cognitive functioning can be particularly enhanced [181, 182]. Additionally, it has been suggested that the structural and functional benefits of exercise can even mimic those of antidepressants [183, 184]. Thus, a prominent role of physical activity in general body and brain health augmentation has been developed.
Finally, substances such as caffeine, widely used in a daily basis, correspond to environmental factors that may directly affect health. Mainly consumed in order to promote alertness and increase wakefulness, caffeine leads to very important secondary effects

on many classes of neurotransmitters, after blocking adenosine receptors, influencing in turn a large number of different physiological functions [185]. Lifelong caffeine intake has been associated with prevention of cognitive decline, and reduced risk of developing stroke, Parkinson's disease and Alzheimer's disease [186, 187, 188, 189, 190]. Caffeine, therefore, being regularly consumed, might serve body and brain health enhancement qualities.
Considering the amount of time that every individual is directly or indirectly affiliated with these environmental factors, delving into their features and actions might prove beneficial. Therefore, environmental factors, such as the ones addressed, to which daily exposure has been noted, may assist in promoting general health and healthy body and brain aging.

1.4 Aim of the current thesis

The current thesis aims to shed light on long-term effects of environmental factors on sleep and the sleep EEG, in the mouse model. *Diet, physical activity, light levels and caffeine intake*, comprise considerable environmental factors in daily life. Investigating the beneficial or adverse effects following long-term exposure to them not only during early adulthood but also in aging provides significant insights that could accelerate, hinder or ameliorate parts of the aging process allowing for a healthier and longer life span.
This thesis is partitioned into two parts, namely the young and the aging part. In particular, **Chapters 2, 3, 4** concern studies conducted in young mice, whereas **Chapters 5, 6, 7, 8, 9** studies conducted in aged mice.
Obesity is associated with sleep disturbances that may interfere with general life quality. Therefore, in **Chapter 2** sleep regulation is studied following diet-induced obesity in young mice. In order for obesity to be developed in mice, so that it simulates the human condition, mice are fed for 12 weeks exclusively with high-caloric diet. The effect of high-caloric diet on sleep homeostasis is tested with the use of parameter estimation analysis and simulations of the sleep homeostatic process S.
In **Chapter 3** the influence of caffeine consumed either acutely or chronically (for two weeks continuously) on sleep, the sleep EEG and behavior is studied in young mice. Although the effects of acute caffeine intake are largely known, inducing almost instantly alertness, caffeine is usually consumed over a longer period of time, and this latter condition has not received much attention from researchers. Thus, in this study caffeine is diluted in the water in order for the mice to have continuous access to it for at least two weeks.
Dim-light-at-night interrupts sleep and circadian rhythms. Therefore, **Chapter 4** focuses on the impact of acute and long-term exposure to dim-light-at-night on sleep, the sleep EEG and behavior in young mice. Particularly, mice are exposed to one night, one week, one month and three months to a light:dim-light-at-night 12:12 h schedule.
Chapter 5 comprises the first of the series of chapters concerning sleep studies in aging. Therefore, in this chapter, sleep and the sleep EEG as well as the morphology of sleep slow-waves is studied in the course of aging in naturally aged mice. The basic detailed effects of aging are hence established, so that the impact of environmental factors in the

chapters that follow can be investigated.
In **Chapter 6** the effect of long-term physical activity on sleep, sleep EEG, and behavior is studied in young and older mice. Exercise, generally, has been proposed to be an alternative to pharmacological treatment for sleep disorders. Hence, in this study, a running-wheel is available in the cages of the mice for one to three months prior to sleep recordings. The sleep recordings are performed with the absence of the running wheel, studying this way the sustained effect of exercise in the course of aging.
In **Chapter 7** the influence of diet-induced obesity on sleep is studied in aged mice. 18 and 24 months old mice are fed exclusively with high-caloric diet for continuous three months prior and during the sleep recordings. This study, therefore, investigates potential obesity-related sleep disturbances in older mice which are eventually compared with the effects in young mice studied in **Chapter 2**.
Chapter 8 investigates the effects of long-term dim-light-at-night exposure on behavior, sleep and the sleep EEG in aged mice, emphasizing the differences from the young mice studied in **Chapter 4**. Thus, 18 and 24 months old mice are exposed for continuous three months to a light:dim-light-at-night schedule.
In **Chapter 9** sleep and the sleep EEG is studied in a genetic mouse model. The main characteristic of this mouse model is the premature aging due to a gene elimination which has a function in DNA repair, rendering the life span of mice to maximum 18 weeks. The data emerging from this mouse model are subsequently compared to data obtained from naturally aged mice, in order to investigate whether this model can produce similar results and eventually being able to replace the natural aging procedure in aging research.
In **Chapter 10** data from all the studies performed are discussed, addressing the main issue of the present thesis, can health in the course of aging be enhanced?

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Part 1

Young data

Chapter 2

High-caloric diet and sleep homeostasis in mice

Chronic high-caloric diet modifies sleep homeostasis in mice

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Abstract

Obesity prevalence and sleep habit changes are commonplace nowadays, due to modern lifestyle. A bidirectional relationship likely exists between sleep quality and metabolic disruptions, which could impact quality of life. In our study, we investigated the effects of a chronic high-caloric diet on sleep architecture and sleep regulation in mice. We studied the effect of 3 months high-caloric diet (HCD, 45 % fat) on sleep and the sleep electroencephalogram (EEG) in C57BL/6J mice during 24-hr baseline (BL) recordings, and after 6-hr sleep deprivation (SD). We examined the effect of HCD on sleep homeostasis, by performing parameter estimation analysis and simulations of the sleep homeostatic Process S, a measure of sleep pressure, which is reflected in the non-rapid-eye-movement (NREM) sleep slow-wave-activity (SWA, EEG power density between 0.5 and 4.0 Hz). Compared to controls (n = 11, 30.7 $\pm$ 0.8 g), mice fed with HCD (n = 9, 47.6 $\pm$ 0.8 g) showed an increased likelihood of consecutive NREM-REM sleep cycles, increased REM sleep and decreased NREM sleep EEG SWA. After SD, these effects were more pronounced. The simulation resulted in a close fit between the time course of SWA and Process S in both groups. HCD fed mice had a slower time constant (Ti = 15.98 hr) for the increase in homeostatic sleep pressure compared with controls (5.95 hr) indicating a reduced effect of waking on the increase in sleep pressure. Our results suggest that chronic HCD consumption impacts sleep regulation.

Keywords: EEG, SWA, sleep regulation, sleep deprivation

Introduction

Modern society is characterized by an increase in obesity prevalence, and concurrently by alterations in sleep habits. A negative relationship between sleep duration and quality, and risk for metabolic disruptions and obesity has been established [1, 2, 3, 4, 5], verifying the role of sleep in the regulation of endocrine functions, glucose metabolism and energy homeostasis.

However, the relationship between sleep, high-caloric diet (HCD) and obesity is likely bidirectional. Obesity is associated with daytime sleepiness and has been characterized as a significant risk factor for sleep disturbances, independent of sleep disorder breathing and age [6]. As obesity is also associated with a widerange of chronic diseases [7, 8], multiple animal models have been developed to study the interaction between sleep and excess body weight.

The majority of these animal models consisted of gene knockout animals, like ubiquitin knockout mice (Ubb-/-) and narcoleptic mice [9, 10, 11, 12] spontaneous mutations in genes like leptin-deficient (ob/ob) or Db/db mice and Obese Zucker rats [13, 14, 15, 16], polygenic models like the OP/OR rat model [17]. Only a rather small amount of studies was conducted on diet-induced obese mice or rats [18, 19, 20, 21], a condition probably closer related to human obesity. Several characteristic changes in sleep noted in humans with obesity were found in these models, for example, increased sleep especially during the active phase, and changes in waking, NREM and REM sleep episode number and duration throughout the 24 hr [4].

In adult humans, the circadian distribution of sleep is monophasic, whereas in rodents, it is polyphasic. Despite this difference, the main homeostatic, circadian and neurochemical modulations of sleep remain essentially similar among species [22]. We consider sleep to be regulated by two main processes, a circadian process governed by the internal biological clock, and a sleep homeostatic process which is dependent on prior waking and sleep [23, 24]. In mammals, the homeostatic sleep process is thought to be reflected in the NREM sleep electroencephalographic (EEG) slow-wave activity (SWA), which is the EEG power density between 0.75 and 4.0 Hz [23, 24]. The dynamics of sleep regulation have been successfully simulated with the use of the two-process model, for the human monophasic sleep-wake pattern [25], as well as for the rodents' polyphasic one [26, 27, 28, 29, 30].

In the aforementioned diet-induced obese models, the regulation of sleep has not been studied. By conducting sleep deprivation, the system is put under elevated homeostatic sleep pressure to better assess the regulation of sleep. This is strengthened by applying parameter estimation analysis and mathematical modeling of the observed homeostatic sleep response visible in the EEG SWA, which is directly associated with the homeostatic sleep process.

We, therefore, investigated the effect of chronic HCD exposure (12 weeks) on baseline (BL) sleep architecture and electroencephalogram (EEG) parameters, as well as after 6 hr of sleep deprivation in C57BL/6J mice. In addition, we performed parameter estimation analysis and simulation of the sleep homeostatic Process S, during the 48-hr recording period, in order to test whether the time course of SWA can be predicted on the basis of temporal organization of sleep. We found altered sleep patterns in mice fed with chronic HCD compared with controls, reflected mainly in increased REM sleep, changes

in absolute SWA and a modulated response to SD. The differences in the dynamics of the SWA levels were consistent with changes found in the estimated time constants of Process S, illustrating first the bidirectional relationship between obesity and sleep, and second, a slower build-up of sleep pressure induced by HCD, leading to an altered sleep homeostasis.

Materials and Methods

Animals

Young male C57BL/6JOlaHsd mice (6 months old; n = 20) (Harlan, Horst, the Netherlands) were used for this study. The C57BL/6 mouse strain is known to be vulnerable to altered dietary patterns, for example high-fat diet, where it induces obesity, hyperglycemia, hyperinsulinemia [31, 32], rendering it an appropriate model to study the effects of diet, obesity and metabolic syndrome. At the age of 3 months, mice were partitioned into two groups: the control group, in which mice were fed with normal chow (11% fat, 27% protein, 61% carbohydrate, Special Diet Services, UK) and the HCD fed group, in which mice were fed exclusively with high-caloric food (45% fat mainly derived from lard, 35% carbohydrate, 20% protein; D12451, Research Diet Services, The Netherlands) for 12 weeks. By providing mice with a chronic HCD diet, we attempted to simulate the human condition as closely as possible, including anatomical (weight gain and body composition) and physiological changes in endocrinology and metabolism, which are reported in the literature [31, 32]. The well-being of all mice was controlled for potential side effects of the diet, and it was ascertained that the animals did not develop any movement problems. The mice were individually housed under controlled conditions (12:12-hr light:dark cycle; lights on at 10:00) with food and water *ad libitum* in a temperature controlled room ($\sim 23°C$).
All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Centre (the Netherlands) and were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Surgeries

On the day of the surgery, control animals (n = 11) weighed on average 30.7 g ($\pm$ 0.8), whereas HCD fed animals (n = 9) weighed 47.6 g ($\pm$ 0.8), showing a 65% increase in body weight compared with controls ($p < 0.0001$). Under deep anesthesia (Ketamine 100 mg/kg; Xylazine 10 mg/kg; and Atropine 1 mg/kg), EEG recording screws (placed above the somatosensory cortex and cerebellum) and electromyogram (EMG) electrodes (placed on the neck muscle) (Plastics One, Roanoke, VA, USA) were implanted as described previously [30, 33]. The wire branches of all electrodes were set in a plastic pedestal fixed to the skull with dental cement. The mice were allowed to recover for 7-10 days.

EEG and EMG recordings

The EEG and EMG were recorded with a portable recording system (PS 1 system, Institute of Pharmacology and Toxicology, Zurich, Switzerland) as previously described [30, 33, 34]. Before each recording, a calibration signal (10 Hz sine wave 300 μV peak-to-peak) was recorded on the EEG and EMG channels. Both signals were amplified, conditioned by analogue filters and sampled at 512 Hz. The signals were filtered through a digital finite impulse response filter and stored with a resolution of 128 Hz. EEG power spectra were computed for consecutive 4-s epochs by a FFT routine within the frequency range of 0.25-25.0 Hz.

Data analysis and statistics

To record the EEG and EMG, animals were placed into experimental chambers and connected through a flexible cable and a counterbalanced swivel system to the recording setup, where conditions were similar to the home cage. Before starting the experiment, animals were allowed to adjust to the experimental conditions for a week. Subsequently, a BL day was recorded, starting at lights on. At the start of the second day, six hours of SD, were conducted by gentle handling, which is a mild intervention in order to induce elevated sleep pressure conditions [27, 30, 33]. EEG and EMG were recorded continuously during SD and, subsequently, for 18 hours to investigate sleep characteristics after SD.
Three vigilance states (Waking, NREM sleep and REM sleep) were scored offline in 4s epochs by visual inspection of EEG and EMG signals as described previously [27, 30, 33, 35]. For each epoch, the EEG power density in the delta (0.75-4.0 Hz) and theta band (6.25-9.0 Hz) and the integrated EMG value were graphically displayed on a PC monitor to enable scoring of the different vigilance states. “Waking” was identified by high EMG and low EEG amplitude as well as high theta activity concomitant with highest EMG values, “NREM sleep” by low EMG and high EEG amplitude as well as high delta activity, and “REM sleep” by low EMG and low EEG amplitude as well as high theta activity. The scores for each 4-s epoch were entered into the PC via the keyboard. Epochs in which the vigilance state could not be identified were excluded and epochs that contained EEG artifacts were marked and excluded from spectral analysis. Subsequently, the vigilance states were expressed as a percentage of artifact-free recording time. The number and duration of the episodes for each state were computed according to the scoring results. Light and dark (L1, D1, L2, D2 for light and dark periods of the first, BL, and second day respectively, after SD) mean values of vigilance states, state episode frequency and duration, transition probabilities and probabilities of repeated NREM-REM sleep cycles' (NRc) were analyzed by three-way repeated measures analysis-of-variance (rANOVA) with main factors ‘treatment’ (between-subject factor), ‘day’ and ‘Light-Dark’ (within-subject factors) to test the effect of SD, and, consequently, by two-tailed unpaired t-tests for each period to determine the effect of treatment. Note that L2 corresponds to the 6-h after SD and is compared to the corresponding 6-h of the BL. An SD effect was considered when an interaction or the factor ‘day’ was significant. The factor‘treatment’ generally refers to the effect of diet. 24-h mean values of vigilance states were analyzed by two-tailed unpaired t-tests. To test the effect of treatment and SD, 2-h values of vigilance states were analyzed by three-way repeated measures ANOVA with main factors

'treatment' (between-subject factor), 'day' and 'time of day' (within-subject factors). To compare NREM sleep EEG power density between the groups across three frequency bins (0.5-4 Hz, 6-9 Hz and 15-25 Hz) two-way ANOVA was performed (main factors 'treatment' and 'frequency'). To test the effect of SD, three-way ANOVA (a repeated measures experimental design was not used due to missing values in both groups across different timepoints) was performed (main factors 'treatment', 'day' and 'time of day') for each frequency bin. Regarding the simulation, two-way repeated measures ANOVA was performed (main factors 'time of day' and 'simulation') for the two experimental groups. The time constants (Ti, Td) and the initial value (iV) were tested with two-tailed unpaired t-tests to determine the effect of treatment. When appropriate (if interaction or main factor effects were significant), post hoc two-tailed paired and unpaired student's t-tests with Bonferroni correction for multiple comparisons were applied to determine the effects of SD or treatment. Correlation coefficient r-values were averaged after Fisher-Z transformation.

Transition probabilities

Transition probabilities were calculated on the basis of frequencies of vigilance state episodes, counted for each mouse individually (Table 1), as it was described previously [36]. Transition probabilities (p) were calculated from the following formulas (#W, #N, #R for the number of Waking, NREM and REM sleep episodes respectively and $p_{W\Rightarrow N}, p_{N\Rightarrow R}, p_{N\Rightarrow W}, p_{R\Rightarrow W}, p_{R\Rightarrow N}$ the transition probabilities between Waking (W), NREM (N) and REM (R) sleep states). It is considered that each waking episode is followed by a NREM sleep episode, rendering this transition probability 100% and that, since REM sleep is preceded only by NREM sleep, the probability for REM sleep to occur is equal to the number of REM sleep episodes (#R) divided by the number of NREM sleep episodes (#N):

$$p_{W\Rightarrow N} = 100\%(1)$$
$$p_{N\Rightarrow R} = \#R/\#N(2)$$
$$p_{N\Rightarrow W} = 1 - \#R/\#N(3)$$
$$p_{R\Rightarrow W} = (\#W - \#N * p_{N\Rightarrow W})/\#R(4)$$
$$p_{R\Rightarrow N} = 1 - p_{R\Rightarrow W}(5)$$

Simulation

We simulated the time course of Process S iteratively on the basis of the vigilance states, as described previously [26, 27, 28, 29, 30]. In the 4-s epochs scored as waking or REM sleep, S increases as a saturating exponential function with an upper asymptote of 1, while in NREM sleep S decreases as an exponential function with a lower asymptote of 0. Time constants for the decrease (Td) and increase (Ti) were determined for each mouse separately, on the basis of vigilance states of the 24-h BL period, 6-h SD and 18-h recovery period. S was computed according to:

increasing function:

$$S_{t+1} = 1 - (1 - S_t) \cdot e^{-\Delta t/Ti} (1)$$

decreasing function:

$$S_{t+1} = S_t \cdot e^{-\Delta t/Td} (2)$$

where t=4-s intervals, $\Delta t = 4 - s, S_t$ and S_{t+1} values of S for consecutive epochs, and Ti and Td the time constants of the increase and decrease rate of S, respectively. The time constants Ti and Td and the initial value of S (iV=S0) were estimated by optimizing the linear correlation between the hourly values of SWA in NREM sleep and S, in BL and recovery for each animal separately. To test whether the estimated parameters could predict the time course of SWA, a simulation was performed over the entire data set consisting of BL, 6-h SD which immediately followed the BL day, and recovery. The optimized parameters of each individual animal were applied to obtain average curves of process S. To enable the comparison between SWA and S, SWA was linearly transformed according to a linear regression based on the 1-h values.

Results

Vigilance states

During undisturbed BL, mice fed HCD showed increased REM sleep during the light period, as compared to control mice, whereas no differences were found in the dark period and only a trend was found in the 24-h values (Fig.1) [REM sleep, post-hoc unpaired t-test for L1, (t,df)=(2.46,72); p=0.016 following significant factor 'treatment' and 24-h value: (t,df)=(1.97,18); p=0.065] (Table 2). No further alterations were detected between the groups in the 12h or 24h NREM sleep and waking values (p>0.05). Compared to BL, decreased waking and increased REM sleep were apparent during the 12-h dark period after SD in the control mice (Fig. 1) [post-hoc two-tailed paired t-tests between BL and after SD time following significant factors 'day' and 'Light-Dark'] (Table 2). In HCD fed mice, decreased waking, as well as increased NREM sleep in both light and dark periods and increased REM sleep in the 12-h dark period were found [post-hoc two-tailed paired t-tests between BL and after SD time following significant factors 'day' and 'Light-Dark'] (Table 2). Differences in the 2-h values of the continuous recordings were mainly evident in REM sleep, where after SD they were more pronounced between the groups, and, additionally, in both groups an effect of SD was apparent on the 2-h values (Fig. 2) [post-hoc two-tailed unpaired t-tests between groups following significant interaction as well as factor 'treatment' and paired t-tests between BL and after SD time following significant factors 'day' and 'Light-Dark'] (Table 2).

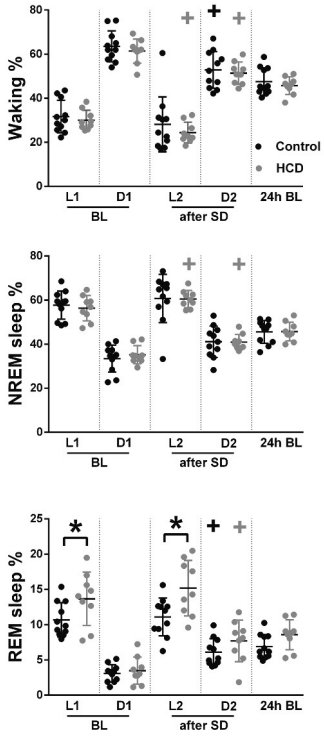

Figure 2.1: Light-dark distribution of each behavioral state (Waking, NREM and REM sleep) during the baseline day (BL: L1 and D1) and after sleep deprivation (after SD: L2 and D2). Scatter plots represent mean (± SD) values (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively) and 24-h values of baseline recordings (24-h BL) for Waking, NREM and REM sleep for control (n=11, black) and high-caloric diet fed mice (HCD, n=9, gray). Asterisks indicate significant differences between the groups (unpaired t-tests for each period, $p<0.05$, after significant repeated measures ANOVA, main factors 'treatment', 'Light-Dark', 'day') and black and gray plus symbols indicate significant differences between recovery and BL day for control and HCD fed mice respectively (post-hoc paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant repeated measures ANOVA, main factors 'treatment', 'Light-Dark', 'day').

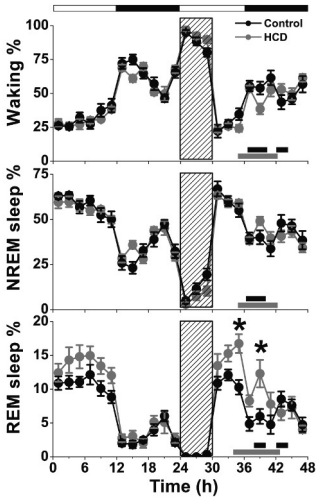

Figure 2.2: Time course of vigilance states, for 24-h baseline (BL), 6-h sleep deprivation (SD, hatched bar) and 18-h recovery for the two groups, control (black circles, n=11) and high-caloric diet (HCD) treated mice (gray circles, n=9). Curves connect 2-h values of Waking, NREM and REM sleep. The black and white bars above each graph indicate the light-dark cycle. Black asterisks at the top of each graph represent significant differences between the groups and black (control) and gray (HCD) bars at the bottom of each graph significant differences between recovery and BL day (post-hoc unpaired and paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant repeated measures ANOVA, main factors 'treatment', 'time of day', 'day').

Episode duration and frequency

We found that diet-induced obesity influenced the 12-h average light and dark NREM and REM sleep episode duration and frequency (Fig. 3). In HCD fed mice, REM sleep episodes were more frequent in both light periods, and they lasted significantly shorter in both dark periods [Frequency, post-hoc unpaired t-test following significant factor 'treatment' for L1: (t,df)=(2.8,72); p=0.0067, L2: (t,df)=(3.8,72); p=0.0003, D2: (t,df)=(2.4,72); p=0.02, Duration, D1: (t,df)=(2.13,72); p=0.037 D2: (t,df)=(2,72); p=0.0485] (Table 2). Under HCD, NREM sleep episodes lasted significantly longer in the BL dark period compared to control [Duration, post-hoc unpaired t-test following significant interaction, D1: (t,df)=(2.43,72); p=0.0176] (Table 2). After SD, HCD fed mice had more NREM sleep episodes in the light period compared to controls [Frequency, post-hoc unpaired t-test following significant factors 'day' and 'Light-Dark' L2: (t,df)=(3.2,72); p=0.002] (Table 2). Compared to BL, SD decreased REM sleep episode frequency in the light period and increased REM sleep episode frequency in the dark period in control animals and increased NREM and REM sleep episode frequency in the dark period in HCD fed animals [post-hoc two-tailed paired t-tests between BL and after SD time following significant factors 'day' and 'Light-Dark'] (Table 2). Additionally, SD shortened waking and NREM sleep episode duration during the dark period and lengthened REM sleep

Table 2.1: Mean numbers (± SEM) of state episodes are shown for Waking, NREM and REM sleep, counted for light and dark periods (L1, D1, D2 for 12-h periods, and L2 for the 6-h recovery period after sleep deprivation) during the 48-h period of recordings for control and high-caloric diet (HCD) fed mice. Asterisks indicate significant differences between groups (unpaired t-tests for each period, $p<0.05$).

Table 1: Number of vigilance state episodes (per h)			
Control mice	Waking	NREM sleep	REM sleep
L1	20 (1.8)	24 (1.1)	10 (1.1)
D1	16.1 (1.8)	17.5 (1.8)	3.3 (0.5)
L2	16.9 (2.5)	20.8 (2.2)	8.5 (0.8)
D2	17.7 (1.2)	20.1 (1.1)	5.7 (0.7)
HCD mice			
L1	19 (2.5)	26.4 (1.8)	15.2 (2.3)*
D1	13.3 (2.2)	15.3 (1.8)	4.4 (0.8)
L2	13.3 (1)	21 (1.5)	14.1 (1.9)*
D2	17.6 (2.4)	21.8 (1.9)	9.4 (1.4)*

episode duration during the light period in HCD fed mice [post-hoc two-tailed paired t-tests between BL and after SD time following significant factors ‘day’ and ‘Light-Dark’] (Table 2). The state transition probability analysis showed that, in the HCD treated mice, a NREM sleep episode was more likely to be followed by a REM sleep episode and less likely to be followed by a waking episode, particularly after SD (Fig. 4) [post-hoc unpaired t-test following significant factors ‘treatment’,‘day’ and ‘Light-Dark’ for L2: (t,df)=(3,72); p=0.003, D2: (t,df)=(2.28,72); p=0.025] (Table 2). Moreover, more sleep episodes were terminated after one NREM-REM sleep cycle (NRc) in the control mice compared to the HCD fed mice (L1: 82% vs. 73%, D1: 92% vs. 84%, L2: 79% vs. 66% and D2: 88% vs. 79%) [main effects ‘day’ and ‘Light-Dark’] (Table 2). Consequently, the probability that a NRc was followed by a second NRc was approximately two times larger in the HCD fed mice compared to controls (L1: 18% vs. 27%, D1: 8% vs. 16%, L2: 21% vs. 34% and D2: 12% vs. 22%) (Fig. S1). Together the data suggest that sleep consolidation was higher in HCD treated mice.

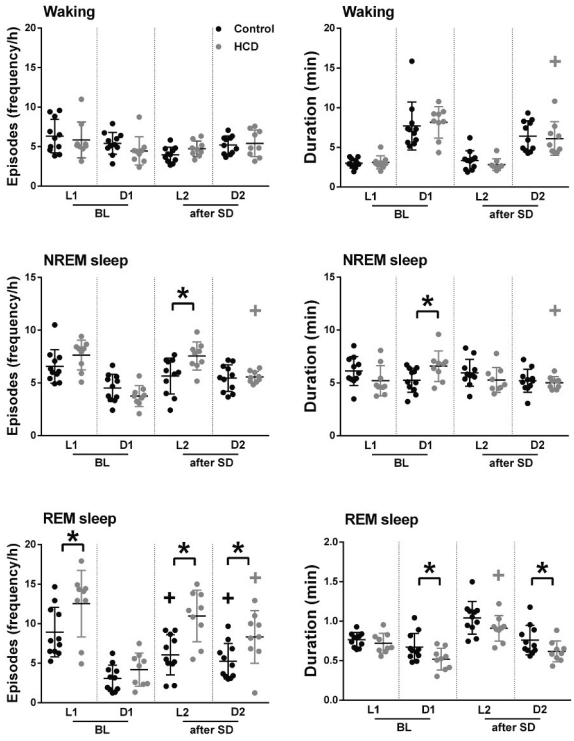

Figure 2.3: Light-dark distribution of episode frequency (frequency/hour, left) and duration (minutes, right) of each behavioral state (Waking, NREM and REM sleep) during the baseline day (BL: L1 and D1) and after sleep deprivation (after SD: L2 and D2). Scatter plots represent mean ($\pm$ SD) values (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively) for Waking, NREM and REM sleep for control (n=11, black) and high-caloric diet fed mice (HCD, n=9, gray). Asterisks indicate significant differences between the groups (unpaired t-tests for each period, $p<0.05$, after significant repeated measures ANOVA, main factors 'treatment', 'Light-Dark', 'day'), and black and gray plus symbols indicate significant differences between recovery and BL day for control and HCD fed mice respectively (post-hoc paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$, after significant repeated measures ANOVA, main factors 'treatment', 'Light-Dark', 'day').

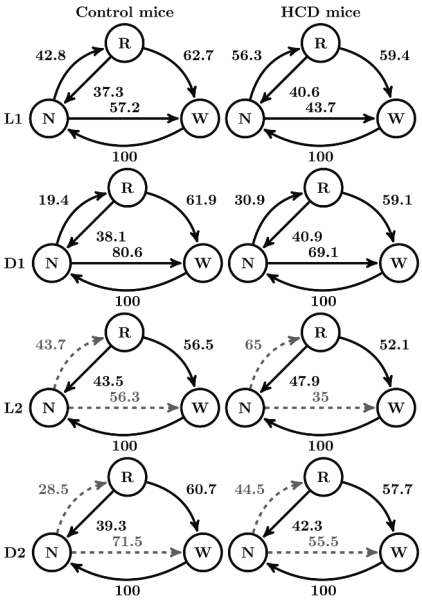

Figure 2.4: State transition probability analysis of the number of vigilance state episode transitions (W = waking, N = NREM sleep, R = REM sleep) for control mice (n=11) and high-caloric diet fed mice (HCD, n=9) (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively). The probability of each state in percent (%) to enter the next state was calculated on the basis of episodes (averages per h shown in Table 1) and is denoted by arrows (see Methods for details). Gray dashed arrows indicate significant differences between the groups (unpaired t-tests for each period, $p<0.05$ after significant repeated measures ANOVA, main factors 'treatment', 'Light-Dark', 'day').

Absolute EEG power density (0.5-25 Hz)

Regarding the spectral analysis of the EEG, differences in the NREM sleep EEG power density between the groups were found across frequency bins (0.5-4 Hz, 6-9 Hz and 15-25 Hz) [Two-way ANOVA, interaction factors 'treatment*frequency'; $F(2,711)=3.7$, $p=0.025$ with main factors 'treatment' $p=0.057$ and 'frequency' $p<0.0001$], which were specific for the SWA range (0.5-4.0 Hz), whereas no differences between the groups were found in the absolute EEG power density levels of 6-9 Hz or 15-25 Hz [post-hoc one-way ANOVA, factor 'treatment'; $F(1,215)=10.4$, $p=0.001$, for the SWA only] (Fig. 5). HCD fed mice had overall significantly lower absolute SWA levels (Fig. 5, top panel). In the first hours after SD, SWA was significantly increased above BL levels in both groups [post-hoc two-tailed paired t-tests between BL and after SD time points following significant factor 'day'] (Table 2). Compared to BL, HCD treated mice showed lower SWA values in the dark period of the recovery day displaying a negative rebound, similar to the negative rebound found previously in the rat [26]. No differences between groups were found in the other frequencies. In both groups, SD induced alterations compared to BL in the theta but not the faster frequencies [post-hoc two-tailed paired t-tests between

BL and after SD time following significant factor 'day'] (Table 2). The absence of differences between the groups in the absolute EEG power density levels of 6-9 Hz or 15-25 Hz (Fig. 5, middle and bottom panels), showed that the effect on SWA was specific for this frequency range and not caused by a general decrease in EEG power density in the HCD fed mice.

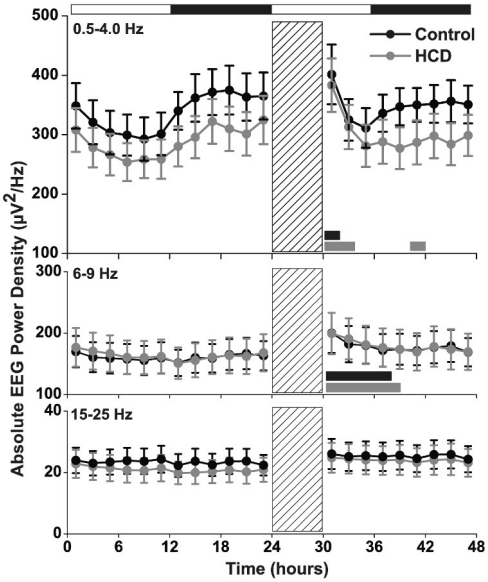

Figure 2.5: Time course of absolute electroencephalographic (EEG) power density ($\mu V^2/Hz$) for the slow-wave activity range (SWA, 0.5-4 Hz) in non-rapid-eye movement (NREM) sleep (upper graph), for 6-9 Hz (middle graph) and 15-25 Hz (lower graph) for 24-h baseline (BL), 6-h sleep deprivation (SD, hatched bar) and 18-h recovery for control (black circles, n=11) and high-caloric diet fed (HCD) mice (gray circles, n=9). Black (control) and gray (HCD) bars at the bottom of each graph indicate significant differences between recovery and BL day (post-hoc paired t-tests with Bonferroni multiple comparisons correction p<0.05, after significant ANOVA main factors 'treatment', 'time of day', 'day'). Overall non-specific decreased EEG SWA levels were revealed in the HCD fed mice compared to control mice, only for the SWA range in BL day (one-way ANOVA, factor 'treatment'; p<0.05).

Simulation of Process S

The differences in sleep-wake distribution and the lower SWA (Fig.1 and Fig.5) suggest that homeostatic sleep pressure may be lower in HCD treated mice. To investigate this further, we simulated the time course of Process S in both groups. The increasing time constant was found to be significantly higher in the HCD fed mice compared to control, whereas no difference was found in the decreasing time constant [HCD mice: Ti=15.98$\pm$0.64, Control mice: Ti=5.95$\pm$0.21; (t,df)=(16.1966,18); p<0.0001, HCD mice: Td=6.22$\pm$0.84 and Control mice: Td=6.0$\pm$0.41; (t,df)=(0.25,18); p=0.81].

The initial value was found to be significantly lower in the HCD fed mice [HCD mice: iV=0.42±0.02, Control mice: iV=0.65±0.02; (t,df)=(9.549,18); p<0.0001]. The simulation of Process S was applied to the 24-h BL, 6-h SD and 18-h recovery, taking into account the parameters estimated for the two consecutive days of recordings of the individuals, and resulted in a close fit between SWA and process S for both groups (Fig. 6) [Control mice: r=0.733, HCD mice: r=0.775]. Discrepancies between the simulation and SWA were found in the light period and after SD for both the HCD fed as well as control mice, similar to an earlier study [Two-way rANOVA, interaction factors 'simulation*time of day', HCD mice: F(41,330)=7.657; p<0.0001 with main factors 'simulation' p=0.919 and 'time of day' p=0.049, Control mice: F(41,407)=7.994; p<0.0001 with main factors 'simulation' p=0.931 and 'time of day' p<0.0001] [27]. The larger increasing time constant in the HCD treated mice indicates that homeostatic sleep pressure increases slower in these animals.

Table 2.2: Elaborate statistical analysis: three-way repeated measures analysis of variance (rANOVA) or ordinary ANOVA was conducted to test the effect of sleep deprivation and high-caloric diet (treatment) (see text for more details). Asterisks following reported p values indicate significance (* p<0.05).

Table 2: Detailed statistics			
Figure	Vigilance state	3-way ANOVA (Interaction factors)	Main factors
1	Waking	'treatment*day*Light-Dark': F(1,18)=0.365 p=0.553	'treatment' p=0.344 'day' p<0.0001 * 'Light-Dark' p<0.0001 *
	NREM sleep	'treatment*day*Light-Dark': F(1,18)=0.511 p=0.484	'treatment' p=0.985 'day' p<0.0001 * 'Light-Dark' p=0.004 *
	REM sleep	'treatment*day*Light-Dark': F(1,18)=0.008 p=0.93	'treatment' p=0.02 * 'day' p<0.0001 * 'Light-Dark' p<0.0001 *
2	Waking	'treatment*day*time of day': F(11,198)=1.704 p=0.075	'treatment' p=0.525 'day' p<0.0001 * 'time of day' p<0.0001 *
	NREM sleep	'treatment*day*time of day': F(11,198)=1.236 p=0.265	'treatment' p=0.672 'day' p<0.0001 * 'time of day' p<0.0001 *
	REM sleep	'treatment*day*time of day': F(11,198)=3.191 p=0.001 *	'treatment' p=0.032 * 'day' p<0.0001 * 'time of day' p=0.001 *
3 [episode frequency]	Waking	'treatment*day*Light-Dark': F(1,18)=0.007 p=0.936	'treatment' p=0.832 'day' p=0.635 'Light-Dark' p=0.042 *
	NREM sleep	'treatment*day*Light-Dark': F(1,18)=0.004 p=0.951	'treatment' p=0.22 'day' p<0.0001 * 'Light-Dark' p=0.033 *
	REM sleep	'treatment*day*Light-Dark': F(1,18)=0.265 p=0.613	'treatment' p=0.012 * 'day' p<0.0001 * 'Light-Dark' p=0.063
[episode duration]	Waking	'treatment*day*Light-Dark': F(1,18)=0.01 p=0.922	'treatment' p=0.91 'day' p<0.0001 * 'Light-Dark' p=0.017 *
	NREM sleep	'treatment*day*Light-Dark': F(1,18)=7.782 p=0.012 *	'treatment' p=0.751 'day' p=0.658 'Light-Dark' p=0.143
	REM sleep	'treatment*day*Light-Dark': F(1,18)=1.03 p=0.324	'treatment' p=0.021 * 'day' p<0.0001 * 'Light-Dark' p<0.0001 *

4	Transition probability NREM-REM sleep	'treatment*day*Light-Dark': F(1,18)=0.312 p=0.583	'treatment' p=0.018 * 'day' p<0.0001 * 'Light-Dark' p<0.0001 *
5	Slow-wave activity (SWA) in NREM sleep	'treatment*day*time of day' : F(11,411)=0.093 p=0.99	'treatment' p<0.0001 * 'day' p=0.028 * 'time of day' p=0.54
	6-9 Hz in NREM sleep	'treatment*day*time of day' : F(11,418)=0.071 p>0.99	'treatment' p=0.902 'day' p=0.032 * 'time of day' p=0.995
	15-25 Hz in NREM sleep	'treatment*day*time of day' : F(11,411)=0.032 p>0.99	'treatment' p=0.223 'day' p=0.093 'time of day' p>0.99
S1	Probability of repeated NREM-REM sleep cycles	'treatment*day*Light-Dark': F(1,18)=0.15 p=0.703	'treatment' p=0.149 'day' p<0.0001 * 'Light-Dark' p=0.006 *

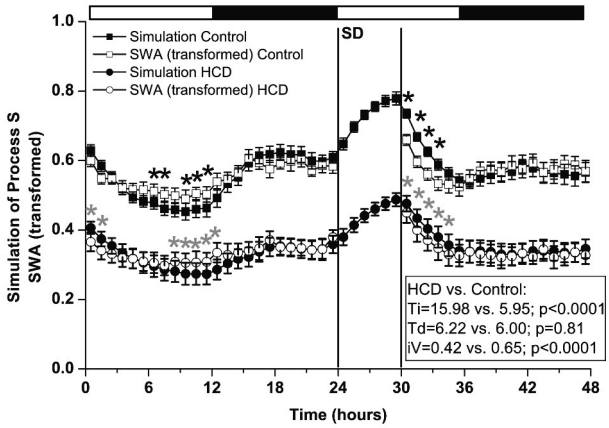

Figure 2.6: Time course of electroencephalographic slow-wave activity (EEG SWA, EEG power density in the range of 0.5-4.0 Hz, linearly transformed according to a linear regression based on the 1-h values) and simulation with the optimized time constants for the increase (Ti), decrease (Td) and initial value (iV) of Process S for the control mice (white and black squares, n=11) and the high-caloric diet fed (HCD) mice (white and black circles, n=9). Curves connect 1-h mean values ($\pm$ SEM) for 24-h baseline (BL), 6-h sleep deprivation (SD) and 18-h recovery. Black and gray asterisks indicate differences between simulation and SWA data for the control and the HCD fed group respectively (paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant repeated measures ANOVA, factors 'simulation', 'time of day'). The optimized mean values of Ti, Td and iV for each condition are noted on the graph along with the p levels (unpaired t-tests, significance when $p<0.05$).

Discussion

In this study, we recorded sleep and the sleep EEG during undisturbed 24-h BL in control mice and mice fed on HCD for three months, and applied a 6-h SD to investigate potential differences in sleep regulation under influence of elevated sleep pressure. We found moderate sleep architecture alterations denoted by an increased REM sleep amount. The results of the sleep episode frequency and duration suggest an increase in sleep consolidation in HCD fed mice. HCD treated mice revealed overall lower absolute SWA levels during NREM sleep, as well as a significant slower increasing time constant for the homeostatic sleep process, compared to control mice. A more pronounced effect on sleep architecture was found after SD, characterized mainly by an increased probability to enter REM sleep from NREM sleep in HCD fed mice, resulting in increased total REM sleep amount compared to control mice, and a negative rebound in SWA level in the dark period, as compared to BL. These changes in SWA were corroborated by changes in the levels of the modeling of the putative average level of the homeostatic sleep process and a slower increase rate in sleep pressure. We conclude that chronic HCD modulates the sleep architecture, although without modifying the daily amplitude of the vigilance state rhythms, and alters sleep homeostasis.

Sleep architecture

Moderate effects of the HCD on sleep architecture were revealed by our results in the BL dark period. Earlier studies showed an increase in the 12-h value of NREM sleep and a decrease in waking during the dark period [19, 20]. These effects were not apparent in our study, however, during the BL dark period, we found longer NREM sleep episodes and shorter REM sleep episodes. Since earlier studies exposed mice to a HCD for a shorter period (usually 8-weeks), a potential explanation for the absence of findings in the amount of waking and NREM sleep in our study could be the longer-term administration of HCD possibly allowing mice to have additional time to adapt to the diet. A clear finding was the increase in REM sleep that was apparent in the HCD fed mice, particularly in the light period compared to control mice, originating from an increase in REM sleep episode frequency, similar to previous studies on HCD in rodents [18, 19, 20, 21]. We conducted further analysis on the number of episodes and showed that an episode of NREM sleep was followed more often by a REM sleep episode in the HCD treated mice, mainly shown after SD, similar to a study in obese compared to lean Zucker rats [14]. Additionally, HCD treated mice revealed an increased probability of an NREM-REM sleep cycle to be followed by a second NREM-REM sleep cycle. Overall these data suggest that sleep consolidation is greater in HCD fed mice compared to controls. The concomitant absence of sleep fragmentation in our data, confirms earlier research in diet-induced obese rodents [18, 19, 20, 37].

The increased transitions into REM sleep can be caused by several different mechanisms. The preoptic area in the hypothalamus is involved in basal metabolism, and has also been associated with REM sleep regulation [38] and thermoregulation [39]. Thermoregulatory control is suspended during REM sleep [40] which is the reason why forced thermoregulatory activation, for instance cold exposure, depresses REM sleep [40, 41]. In contrast, sleeping in thermo-neutral conditions, where active thermoregulation is not necessary, increases REM sleep [42]. Similarly, Djungarian hamsters that reduce body temperature, when exposed to short photoperiods to save energy with reduced thermoregulation, show an increase in NREM-REM sleep transitions when kept at the same ambient temperature, as demonstrated in the present study [36]. In these hamsters, there was a clear relationship between the NREM-REM transition probability and the set point of body temperature. In accordance with this, it was shown that the metabolic efficiency index, an indirect measure for metabolic rate, was lower in high-fat diet-fed mice compared with normal diet-fed mice [43]. Considering the body composition changes in HCD fed mice, which gained weight and have a smaller surface-to-content ratio compared to control mice, it is likely that these mice will cool down slower and will probably show less thermoregulatory activity. This is in congruence with a reduced metabolic rate and an increase in REM sleep episode frequency. Therefore, a mere change in body size and thermoregulatory activity may explain the difference in REM sleep patterns observed.

However, changes in neurotransmitter release, under influence of HCD, may also alter sleep. Obesity has been associated with decreased levels of orexin in humans and mice [44, 45, 46, 47]. Orexin is predominantly found in posterior lateral hypothalamus and is a critical regulator of vigilance states, energy homeostasis, as well as the reward system and feeding [12, 48]. In narcolepsy, which is caused by the loss of neurons that contain orexins or orexin receptors, massive increases in REM sleep are found [49]. Thus,

changes in orexin levels in HCD fed mice may have also influenced sleep architecture in this group. Disentangling the different mechanisms affecting REM sleep in HCD induced obese models may prove to be difficult, as the diet is likely to produce several of these changes in parallel, which may all promote the increase in REM sleep.
We found a remarkably intact day-night modulation of sleep and waking. In other words, the daily amplitude of the vigilance state rhythms did not differ between the two groups, owing to the modest effects of HCD on sleep architecture. HCD is known to disturb circadian organization, particularly in the peripheral clocks, whereas functioning of the central clock seems to remain intact [50]. The present data show only minor changes in the distribution of sleep. This suggests that the day-night distribution of sleep and wakefulness is mainly determined by the central clock in the suprachiasmatic nucleus and only mildly influenced by changes in peripheral clocks.

NREM sleep EEG SWA: data and simulations

An overall decrease in absolute EEG SWA during NREM sleep was revealed in the HCD fed mice compared to control. This decrease was specific for the slow wave range, as other frequency ranges (theta, fast frequency activity) were not affected. Additionally, the absolute SWA levels in the HCD fed mice showed a negative rebound in the dark period after SD, similar to a previous study in rats [26], revealing an altered response to SD. A study in rats showed that increased sleep induced by high-fat/high-salt diet (i.e. cafeteria food) was associated with decreased EEG SWA during several days of cafeteria food [37], similar to our findings. However, studies in C57BL/6 mice found no alterations in EEG SWA compared to control across a 6 or 10-week exposure to HCD experiment [19, 20].
Previous findings of the effect of HCD on sleep and the sleep EEG were not always consistent. In our data, we found lower SWA and increased REM sleep that indicate lower homeostatic sleep pressure. In order to elucidate these findings, we further analyzed the effect of HCD on the regulation of sleep by modeling the homeostatic sleep response. We found a significantly higher increasing time constant and a lower initial value of S in the HCD fed mice. The time constants obtained for the two groups could predict the effect of SD, with similar discrepancies between the two groups, consistent with the study of Huber et al. (2000). The discrepancies, as previously indicated, may emerge from the mathematical limitations of the model adjusted for mice data [27]. Importantly, the time constants of the control mice were similar to previous estimations in the C57BL/6J mouse strain [27]. However, the significantly higher increasing time constant in the HCD fed mice (Ti= 15.98h) suggests that the build-up of sleep pressure during waking and REM sleep is slower in these mice. On the contrary, the decrease rate obtained from our data, is similar between the groups, resembling previous reported decrease rates in mice [27], and remains unaffected by the HCD. The decreasing time constant is unaltered among different species, as indicated in mice, rats and humans [27], and it seems that it likely obtains more rigid properties. Therefore a condition such as obesity is probably not expected to alter it significantly. Thus, sleep homeostasis seems to be influenced by HCD, rendering these mice less susceptible to prolonged waking.
The mechanisms underlying HCD influence on the occurrence of SWA or sleep homeostasis remain elusive. High-fat feeding and food restriction are thought to enhance ke-

togenesis [51]. Ketone bodies are generated from the breakdown of fatty acids and have been shown to become major fuels in most tissues during starvation, prolonged exercise, or consumption of a high-fat, low-carbohydrate diet [52]. Ketogenic diet has been applied as a treatment for epilepsy, autism and brain tumours, and it has been shown to induce sleep alterations [51] possibly by a shift of the excitatory/inhibitory (E/I) balance in the cortex to a more inhibitory state [53], which is consistent with the lower SWA found in our analysis [54]. Although less extreme than a pure ketogenic diet, HCD may have an effect on sleep homeostasis through the fatty acids metabolic pathway. Additionally, changes in melanin concentrating hormone (MCH), an appetite-stimulating peptide expressed in the lateral hypothalamus, shown to have an increased expression in adult offspring after prenatal HCD exposure [55], may also mediate SWA and/or sleep homeostasis alterations, since MCH neurons were proposed to be implicated in sleep-wake regulation [56]. In conclusion, multiple pathways are likely to be involved in the case of obesity considering that sleep is governed by complex brain networks. Further research is, hence, needed to elucidate the mechanisms underlying the HCD effects on sleep homeostasis.

Concluding Remarks

In the current study we show that, although sleep architecture is not strongly affected after chronic HCD, sleep homeostasis and the response to SD is altered. Translated to humans, we could deduce that HCD reduces the effect of prolonged waking on subsequent EEG SWA in NREM sleep. Prolonged waking is known to increase craving for food, especially for high-fat and high-carbohydrate food [57]. This change in diet may have a similar effect on the build-up of sleepiness as caffeine [58], however not by influencing adenosinergic mechanism, but by a change in the E/I balance of neuronal activity in the cortex. Therefore, the increased craving for high-caloric food when sleep deprived may have short-term benefits by lowering the burden of the sleep debt incurred. Nevertheless, a vicious cycle is likely created, in which high caloric intake and prolonged waking strengthen each other, inducing an increase in weight with all the associated health risks. That way, HCD and reoccurring sleep debt are possibly synergistic entities, that in the long-term are detrimental for health and longevity.

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Author Contributions Statement

T.DB. and J.H.M. designed research; M.P. and T.DB. performed research; M.P. and T.DB. analyzed data; and M.P., J.H.M., and T.DB. wrote the paper.

Competing financial interests

The authors declare no conflict of interest.

Data Accessibility Statement

The authors deposit supporting information and datafiles to Figshare for data archiving https://figshare.com/s/f2db8ab6be1ee1b9e875

Abbreviations

BL, baseline; EEG, electroencephalogram; E/I, excitatory/inhibitory; EMG, electromyogram; HCD, high-caloric diet; NREM, non-rapid-eye-movement; SWA, slow-wave activity (EEG power density in NREM sleep between 0.75-4.0 Hz); SD, sleep deprivation.

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Supplementary Material

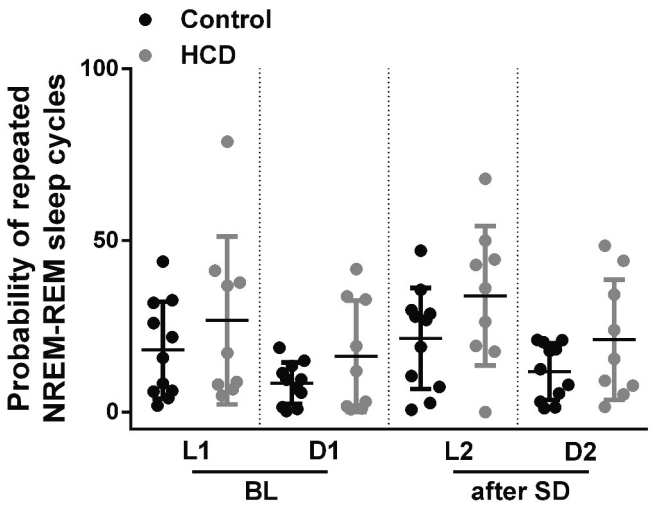

Figure 2.1: Probability of repeated NREM-REM sleep cycles (NRc) for control (n=11) and high-caloric diet fed mice (HCD, n=9) (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively). The probability that a NRc was followed by a second NRc was approximately two times larger in the HCD fed mice compared to controls (repeated measures ANOVA, main factors 'treatment' ($p=0.149$), 'Light-Dark', 'day' $p<0.05$).

Chapter 3

Chronic caffeine consumption and sleep: a paradox?

Effects of chronic caffeine consumption on sleep and the sleep electroencephalogram in mice

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Abstract

Background: Caffeine is one of the most widely consumed psychostimulants, and it impacts sleep and circadian physiology.
Aim: Caffeine is generally used chronically on a daily basis. Therefore, in the current study, we investigated the chronic effect of caffeine on sleep in mice.
Methods: We recorded the electroencephalogram and electromyogram on a control day, on the first day of caffeine consumption (acute), and following two weeks of continuous caffeine consumption (chronic). In the latter condition, a period of six-hour sleep deprivation was conducted during the light period. Control mice, which received normal drinking water, were also recorded and sleep deprived.
Results: We found that caffeine induced differential effects following acute and chronic consumption. Over 24 h, waking increased following acute caffeine whereas no changes were found in the chronic condition. The daily amplitude of sleep–wake states increased in both acute and chronic conditions, with the highest amplitude in the chronic condition, showing an increase in sleep during the light and an increase in waking during the dark. Furthermore, electroencephalogram slow-wave-activity in non-rapid eye-movement sleep was increased, compared with both control conditions, during the first half of the light period in the chronic condition. It was particularly challenging to keep the animals awake during the sleep deprivation period under chronic caffeine.
Conclusions: Together the data suggest an increased sleep pressure under chronic caffeine. In contrast to the traditional conception on the impact on sleep, chronic caffeine intake seems to increase the daily sleep–wake cycle amplitude and increase sleep pressure in mice.

Keywords: Caffeine, electroencephalogram, mice, sleep

Introduction

Caffeine is a psychoactive stimulant that is commonly used worldwide. It mainly acts as a nonselective adenosine receptor antagonist, disrupting sleep and increasing alertness in mammals [1, 2, 3]. At doses generally consumed by humans, caffeine produces its arousing effect by partial nonselective blockade of adenosine 1 and adenosine 2A receptors [4, 3, 5].

Sleep is considered to be regulated by two main processes [6, 8]. The timing of sleep is regulated by the circadian pacemaker, which in mammals is located in the suprachiasmatic nucleus (SCN) of the hypothalamus [7]. The depth of sleep is homeostatically regulated, with increasing sleep propensity during waking and dissipation of sleep pressure during sleep. Prolonged waking is compensated by deeper and sometimes longer sleep. In mammals, the homeostatic sleep process is reflected in electroencephalogram (EEG) slow-wave activity (SWA; EEG power density below 5 Hz) during non-rapid eye-movement (NREM) sleep [6, 8, 9], with higher SWA representing deeper sleep or higher homeostatic sleep pressure.

Adenosine release in the brain shows a positive correlation with the previous amount of waking [10, 11], and adenosine administration induces deep NREM sleep with high amplitude slow-waves [12, 13]. Adenosine is therefore one of the substances thought to be involved in sleep homeostatic regulation [14]. Acute application of caffeine is known to reduce or disturb sleep in a dose dependent manner [15, 16, 17, 18] and it reduces EEG SWA in subsequent NREM sleep in humans and rodents [19, 20, 21, 22, 23, 12]. Recently it was suggested that both acute and chronic caffeine lengthen the circadian period [24, 25, 26, 27] and acute caffeine treatment delays dim light melatonin onset in humans [28]. Therefore, caffeine not only influences sleep homeostatic mechanisms, but probably also the circadian clock.

In most animal studies the effects of caffeine on adenosine-related sleep regulatory mechanisms are investigated by acute administration of caffeine through intraperitoneal (i.p.) injections [12, 27, 29, 30, 31]. Considering that in real life caffeine is used chronically as a stimulant, it is remarkable that sleep polysomnographic studies investigating the effect of chronic caffeine use are rare. Sleep in cats normalised under chronic caffeine, after an initial increase in waking, but deeper stages of sleep remained below baseline levels [32]. One study in humans showed that acute caffeine consumption increased sleep latency and decreased sleep, however, these effects faded out after one week of chronic caffeine consumption [33]. Epidemiological studies support the idea that increased caffeine use is associated with shorter or more disturbed sleep reviewed by [4, 5]. Nevertheless, long-term EEG recordings under chronic caffeine consumption are lacking, being difficult to perform in humans.

To better approach the normal caffeine consumption pattern in humans and investigate the impact of chronic caffeine consumption on sleep, we subjected mice to caffeine in the drinking water for two weeks. Sleep and the sleep EEG were recorded under control conditions, with normal drinking water, during the first day in which the mice received caffeine in the drinking water, and following two weeks of chronic treatment with caffeinated drinking water. In addition, in the chronic condition the mice were sleep deprived for six hours and recovery sleep was analysed. We found that chronic caffeine intake increases the daily light–dark amplitude of the sleep–wake rhythm and deepens

sleep during the rest phase.

Materials and methods

Animals

Twelve-week-old male C57BL/6JOlaHsd mice (Harlan, Horst, The Netherlands) were used in this study. All mice were housed under controlled conditions (12 h:12 h light:dark cycle, lights on at 10:00) with food and water *ad libitum* in a temperature-controlled room (23-24°C). All animal experiments were approved by the Animal Experimental Ethical Committee of the Leiden University Medical Center (LUMC) and were carried out in accordance with the EU directive 2010/63/EU on the protection of animals for scientific purposes.
At the age of 12 weeks, animals were operated under deep anaesthesia and EEG and electromyogram (EMG) electrodes were implanted as described previously [30, 34, 35]. One EEG electrode was placed over the right hemisphere (2 mm lateral to midline of the skull, 2 mm posterior to bregma) above the somatosensory cortex, while the other was placed above the cerebellum (at midline, 2 mm caudal to lambda). The EMG electrodes were placed on the neck muscle (left and right of midline). The wire branches of the electrodes were set in a plastic pedestal (Plastics One, Roanoke, Virginia, USA) which was fixed to the skull with dental cement. The mice were allowed to recover for at least seven days. After the surgery the animals remained single-housed until the end of the experiment.

Drug preparation

Caffeine (LUMC Pharmacy) was dissolved in the drinking water at a concentration of 0.8 mg/mL (0.08%). This concentration was found to have significant effects on the circadian rhythm [26, 27], and is equivalent to the caffeine concentration in ordinary drip coffee. The animals drank approximately 6 mL water per day, both in the caffeinated condition as in the control condition. Before the start of the experiment the mean weight of the animals was 29.6 ($\pm$0.7 standard error of the mean (SEM)) g. Shortly before the animals were taken off the caffeinated water the average weight was 30.1 ($\pm$0.4) g. We do not have any indication that the animals drank or ate different amounts during the period the water was caffeinated.

EEG recordings

The EEG and EMG were recorded with a portable recording system (PS 1 System, Institute of Pharmacology and Toxicology, Zurich, Switzerland) as previously described [30, 34, 35]. Before each recording, a calibration signal was recorded on the EEG and EMG channels. Both signals were amplified ($\sim$2000 times), conditioned by analogue filters and sampled at 512 Hz. The signal was filtered through a digital finite impulse response filter and stored with a resolution of 128 Hz. EEG power spectra were computed for four-second epochs. For the recordings, the animals were placed in the experimental chambers and connected through a flexible cable and a counterbalanced swivel with

the recording system. Conditions in the experimental chamber were similar to the home cage. The animals were allowed to adjust to the experimental conditions for at least a week.
In seven animals a control day was recorded, starting at dark onset. At the start of the second dark period the water bottles were replaced by bottles with caffeinated drinking water (acute caffeine). After two weeks on the caffeinated drinking water, recordings were resumed (chronic caffeine baseline and sleep deprivation), starting at lights on. At the start of the second day, six hours of sleep deprivation were conducted by gentle handling, which is a mild intervention in order to induce elevated sleep pressure conditions [30, 38, 35]. During that period, every time the animals appeared drowsy or the EEG exhibited slow-waves, the animals were mildly disturbed by noise, movement of the bedding, or introducing fresh food or nesting material into the cage. EEG and EMG were recorded continuously during sleep deprivation and the following 18 h to investigate the effects of the sleep deprivation on subsequent sleep and waking. A second set of mice (n=11, controls=2), which were kept on normal drinking water, were recorded under similar conditions and were sleep-deprived in the same way as controls. It should be noted that, although the sleep-deprivation protocol was conducted in a similar way, it was more challenging in the chronic caffeine-treated mice. With a similar number of mice, only one experimenter was needed for the control sleep deprivation, whereas two were occupied with the sleep-deprivation experiment in the chronic caffeine condition, albeit with a less successful outcome.

Behavioural activity

After finalising the EEG and EMG recordings, mice on chronic caffeine were transferred to cages containing passive infrared (PIR) motion detectors (Hugrosens Instruments, Löfflingen, Germany). The PIR detectors were connected to a ClockLab data collection system (Actimetrics, Illinois, USA). The number of PIR counts was measured and stored on a computer in one-minute bins. The mice remained for another two weeks on caffeinated drinking water and were subsequently transferred to normal drinking water for another two weeks. Activity strength was analysed with F-periodograms as previously described [36, 37].

Data analysis and statistics

Three sleep-wake states (waking, NREM sleep, and REM sleep) were scored offline in four-second epochs by visual inspection of the EEG and EMG signals, as well as EEG power density in the slow-wave range, as described previously [30, 34, 35, 38]. Epochs with artefacts were excluded from the subsequent analysis of power spectra, but sleep-wake states could always be determined. Light, dark and 24-hour mean values of sleep-wake states, as well as two-hour values of sleep-wake states and EEG SWA in NREM sleep were analysed. Sleep-wake state episodes were determined with an algorithm as described previously [38, 39].
To test the effect of caffeine, one-way (factor 'treatment') and two-way repeated measures analysis-of-variance (rANOVAs) (factors 'treatment', 'light-dark' or factors 'treatment', 'EEG-frequency' or factors 'treatment', 'time of day') were performed followed by Bonferroni corrected or Tukey's Honest Significant Differences (Tukey's HSD) paired

or unpaired t-tests where appropriate. In the case of statistical analysis including the second control group, in which different animals were used or regarding the EEG SWA analysis due to missing values, a repeated measures experimental design was not used; instead ordinary two- or three-way (factors 'treatment' x 'time of day' or factors 'treatment', 'day', 'time of day') were performed.

Results

Rest-activity behaviour

The influence of caffeine on rest-activity rhythms was investigated by counting PIR activity in one-minute intervals over a period of approximately three weeks (Figure 1). During the first part of the recordings, the animals were provided with caffeinated drinking water, while in the second part with normal drinking water. Under chronic caffeine, the animals showed a slight delay in activity onset and an increase in activity in the first half of the dark period (two-way rANOVA factors 'treatment' $p<0.001$; 'treatment' x'time of day' $p<0.001$). No significant differences were found in total activity counts over 24 h or in the strength of the rhythm ($p>0.1$).

Sleep-wake states

Acute caffeine

Caffeine had pronounced effects on the distribution of sleep and waking over the day (Figures 2 and 3, 12-hour values in Figure 2: two-way rANOVA, interaction factors 'treatment' x'light-dark', waking: $p<0.0001$ and 'treatment' $p=0.0053$, 'light-dark' $p<0.0001$, NREM sleep: $p<0.0001$ and 'treatment' $p=0.0024$, 'light-dark' $p<0.0001$, REM sleep: $p=0.26$. Two-hour values in Figure 3: two-way rANOVA, factor 'treatment' for waking: $p<0.0001$, NREM sleep $p<0.0001$, REM sleep (not significant (n.s.)). Under acute caffeine, waking increased in the dark period, whereas it was not altered in the light period, figures 2 and 3. NREM sleep mirrored this pattern with a decrease in the dark period and no change in the light period. Over 24 h the amount of waking was increased and NREM sleep decreased, reflecting the changes found in the dark period (Figure 2 (a), one-way rANOVA, factor 'treatment', waking: $p=0.0113$, NREM sleep: $p=0.0076$, REM sleep: $p=0.47$). The changes in the dark period resulted in an overall increase in the difference between the light and dark period in the amount of waking and NREM sleep (Figure 2 (b), One-way rANOVA, factor 'treatment', waking: $p=0.0002$, NREM sleep: $p<0.0001$). For the acute caffeine condition, NREM sleep episode duration and REM sleep episode frequency decreased in the dark period, showing a reduced NREM sleep episode consolidation (Table 1).

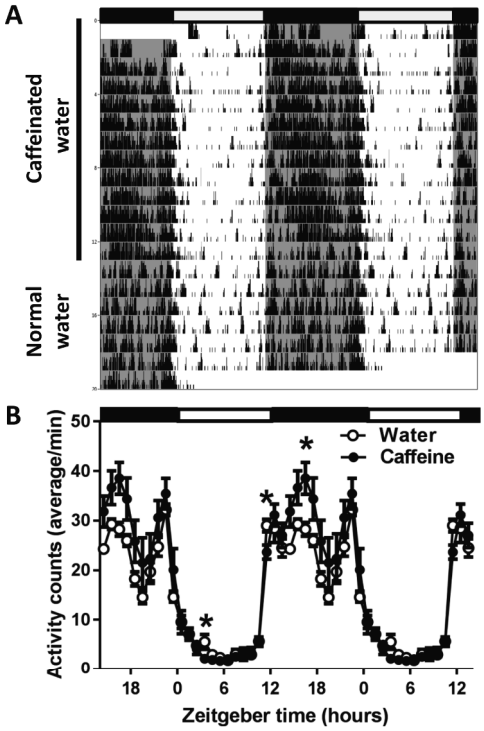

Figure 3.1: (a) A representative example of locomotor activity (passive infrared (PIR) recording) of the activity of a mouse. During the first 13 days the mouse drank exclusively caffeinated water (indicated by the black bar on the left). Subsequently, the bottle was replaced with normal drinking water. Black and white bars at the top indicate the light-dark cycle. (b) Average time course of locomotor activity over the last 10 days of chronic caffeine treatment and the first 10 days after return to normal water (n=7). Curves connect one-hour values (mean±standard error of the mean (SEM)) of locomotor activity recorded with a passive infrared sensor. The black and white bars indicate the light–dark cycle. Asterisks indicate significant differences between the two conditions ($p<0.05$ paired t-test, after significant two-way analysis of variance (ANOVA), factors 'treatment'×'time of day')

Chronic caffeine

Following two weeks of caffeine intake, the distribution of sleep was altered (Figures 2 and 3). Compared with the acute condition, waking during the dark period was decreased and the amount of NREM sleep was increased. However, increased waking and decreased NREM sleep in the dark period prevailed compared with control levels (Figures 2 (a) and 3). Notably, during the light period the opposite effect was found. Interestingly, the 24-hour values of waking, NREM sleep and REM sleep returned to control levels and the amount of waking and NREM sleep differed significantly from the acute caffeine condition (Figure 2 (a)). This resulted in a further increase in the daily amplitude of

NREM sleep (Figure 2 (b)).
Chronic caffeine consumption shortened NREM sleep episodes and reduced transitions into REM sleep in the dark period, similar to the acute caffeine condition (Table 1). This was accompanied by an increase in the number of waking episodes. However, in the light period, there was an opposite effect with an increase in the duration of NREM sleep episodes. The effects found in the dark period were very similar to those found in the acute condition but, in addition, chronic caffeine seemed to increase NREM sleep episode consolidation in the light period.

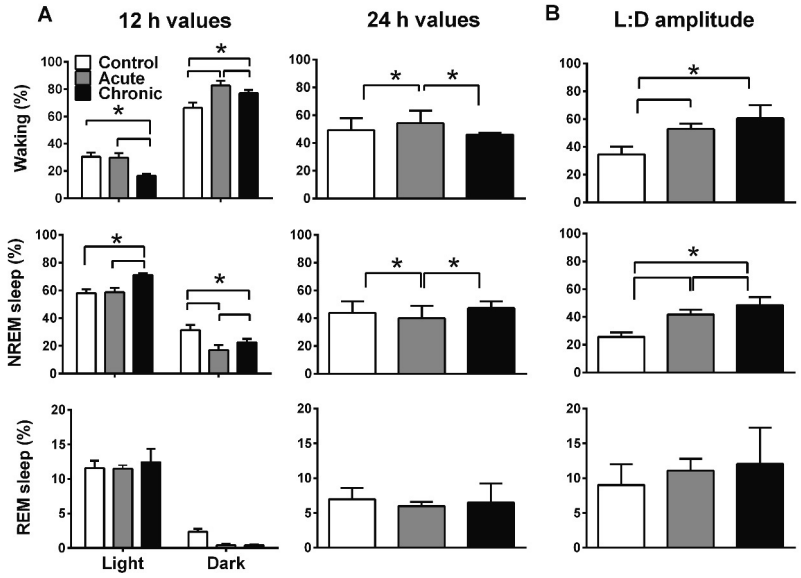

Figure 3.2: (a) Dark, light and 24-hour values of sleep-wake states (n=7). Note that the light and dark data for the chronic caffeine condition are plotted in reverse order of recording to match the order of recording of the control and acute condition. Asterisks indicate significant differences between conditions (post-hoc Tukey's multiple comparisons test after significant two-way repeated analysis of variance (r-ANOVA), factors 'treatment'×'light-dark' or one-way r-ANOVA factor 'treatment' (24-hour values). (b) Light-dark amplitude of sleep-wake states. Asterisks indicate significant differences between conditions ($p<0.05$, post-hoc Tukey's multiple comparisons test after significant one-way r-ANOVA factor 'treatment'). NREM: non-rapid eye movement; REM: rapid eye movement.

Table 3.1: Vigilance state episode frequency/h and duration (min).
*Significantly different from control #Significantly different from acute (p<0.05 paired t-test after significant ANOVA factors 'treatment', 'Light-Dark'). Note that the light and dark data for the chronic caffeine condition are plotted in reverse order of recording to match the order of recording of the control and acute condition.

Table 1. Average 12-h values of vigilance state episode frequency/h and duration (min)						
	Control		Acute		Chronic	
Frequency (/h)	Dark	Light	Dark	Light	Dark	Light
Waking	4.2 (0.9)	6.3 (0.6)	6.1 (1.0)	6.3 (0.3)	6.7 (0.6)*	4.5 (0.3)
NREM sleep	5.2 (0.5)	6.9 (0.5)	5.7 (1.0)	7.0 (0.4)	5.8 (0.6)	6.2 (0.3)
REM sleep	2.2 (0.6)	8.3 (1.0)	0.5 (0.4)*	7.6 (0.3)	0.4 (0.1)*	6.5 (0.9)
Duration (min)						
Waking	8.9 (1.4)	2.9 (0.5)	10.3 (2.4)	2.9 (0.4)	7.7 (1.1)	2.0 (0.2)
NREM sleep	4.3 (0.4)	5.9 (0.4)	2.3 (0.2)*	5.7 (0.4)	2.9 (0.2)*	7.5 (0.4)*#
REM sleep	0.7 (0.1)	0.9 (0.1)	0.8 (0.3)	1.0 (0.0)	0.7 (0.2)	1.2 (0.1)

EEG power density

To investigate whether caffeine had an influence on brain functioning and alertness we investigated the EEG power density spectra of the mice during the different sleep-wake states (Figure 4) (two-way rANOVA, interaction factors 'treatment' x'EEG-frequency', Waking: p<0.0001, NREM sleep: p<0.0001, REM sleep: p=0.0001). The acute effects of caffeine were moderate with a small decrease in power density found around 3 Hz and small increases around 7 and 9 Hz in the NREM sleep EEG. A similar decrease was found around 3-4 Hz in REM sleep. In the waking EEG, power density around 10 Hz was increased. The effects of chronic caffeine consumption were more pronounced, showing a significant decrease in power density below 8 Hz and an increase in the theta range (9-10 Hz) in the waking EEG, and a clear decrease in activity between 3 and 20 Hz in NREM sleep. In REM sleep, decreases were found in the slow-wave range (0.5-5 Hz), between 7-13 Hz, and around 17 Hz.

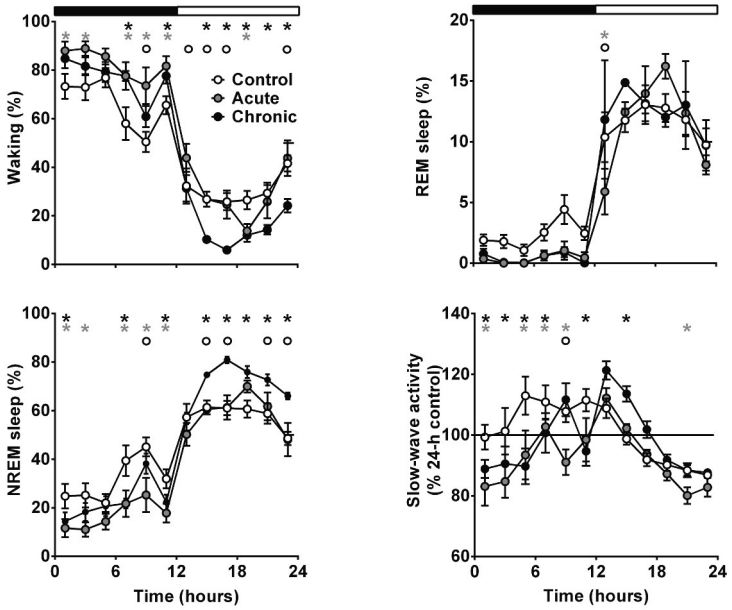

Figure 3.3: Time course of waking, non-rapid eye movement (NREM) sleep, rapid eye movement (REM) sleep, and electroencephalogram (EEG) slow-wave-activity (SWA) in NREM sleep for the 24-hour baseline day in the control condition and during the acute and chronic caffeine conditions (n=7). Note that the light and dark data for the chronic caffeine condition are plotted in reverse order of recording to match the order of recording of the control and acute condition. Curves connect two-hour values (mean±standard error of the mean (SEM)). The black and white bars indicate the light-dark cycle. Asterisks indicate significant differences between acute (grey) or chronic (black) caffeine condition compared with control. The circles indicate significant difference between the acute and chronic caffeine condition ($p<0.05$, Bonferroni multiple comparisons test after significant twoway ANOVA, factors 'treatment' or 'treatment'×'time of day').

NREM sleep slow-wave activity

The redistribution of sleep and wakefulness, with less sleep during the dark period and more sleep during the light period, could affect sleep homeostasis. We, therefore, analysed the time course of EEG SWA during NREM sleep in the three conditions (Figure 3). In the control condition, SWA showed its well-known gradual decrease in the course of the light period and increase during the dark period (two-way ANOVA, interaction factors 'treatment' x 'time of day' $p=0.0002$ and 'treatment', 'time of day' $p<0.0001$). In the acute caffeine condition, SWA levels were lower during several two-hour intervals in the dark period, but they did not differ from control levels during the light period. In the chronic caffeine condition, SWA levels were also lower during the dark period, but in contrast to the acute condition, they were significantly increased above control levels during the light period. This suggests that the animals had a higher sleep pressure and slept more deeply during the first part of the light period.

Effects of sleep deprivation

To test this further, a sleep deprivation was conducted during the first part of the light period, the time where the largest difference was found in SWA between the two groups. These data were compared with an additional group of animals that were provided with normal drinking water. The amount and time course of sleep and waking across the baseline day of this group did not differ from the data obtained from control day of the group subsequently drinking caffeinated water (data not shown).

The comparison of the baseline data between the chronic caffeine group and the second control group confirmed the changes found after application of chronic caffeine described above (three-way ANOVA, with factors 'treatment', 'time of day', 'day' and their interactions, waking: 'time of day', 'day': $p<0.0001$, interaction 'treatment' x 'time of day' $p<0.0001$, NREM sleep: 'time of day' and 'day' $p<0.0001$, and interaction'treatment' x 'time of day' $p<0.0001$, REM sleep: 'treatment', 'time of day' and 'day' $p<0.0001$, and interaction 'treatment' x 'time of day' $p<0.0001$, SWA: 'treatment' $p<0.0001$, 'time of day' $p=0.018$, and interaction 'treatment' x 'day' $p=0.008$). During the light period, waking was decreased and NREM sleep was increased in the chronic caffeine condition (Figure 5). In the dark period, the opposite was found, together with a decrease in REM sleep. In the chronic caffeine condition, SWA was above control levels during the first six hours of the light phase and lower than control levels during the first six hours of the dark phase.

In the course of the sleep deprivation it was noted that, compared with the second control group, it was very difficult to keep the animals on chronic caffeine awake. In the initial two hours the amount of waking did not differ between the two groups. However, the level of waking in the chronic caffeine group dropped significantly below the level of the control group in the remaining four hours. The amount of REM sleep was equally low in both groups during sleep deprivation ($\sim 0\%$). In addition, in both groups SWA in NREM sleep was increased above baseline in the first two hours after sleep deprivation.

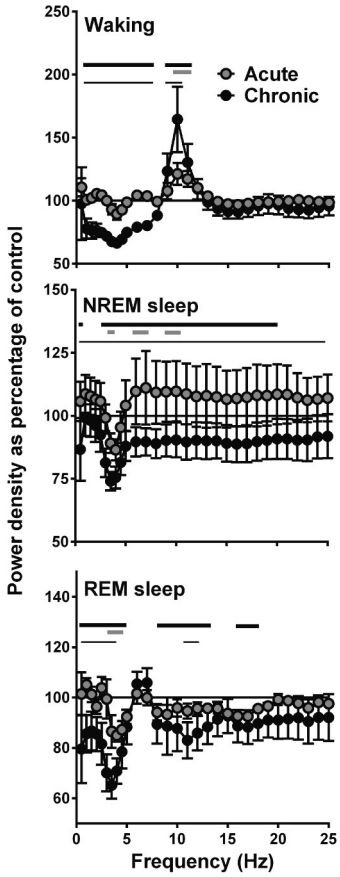

Figure 3.4: Electroencephalogram (EEG) power density in waking, non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep. Between 0.25 and 5.0 Hz, values were calculated in 0.5-Hz bins and between 5.25 and 25.0 Hz in 1-Hz bins (n=7). Values are plotted at the upper limit of each bin. Curves connect 24-hour mean values of relative power density for the acute and chronic caffeine condition relative to the control condition (=100%). Thick lines above the curves indicate frequencies where the chronic caffeine (black) and acute caffeine (grey) conditions differ from control. Differences between the acute and chronic condition are indicated by thin black lines ($p<0.05$, Tukey's multiple comparisons tests after significant two-way repeated analysis of variance (r-ANOVA), factors 'treatment'×'EEG-frequency').

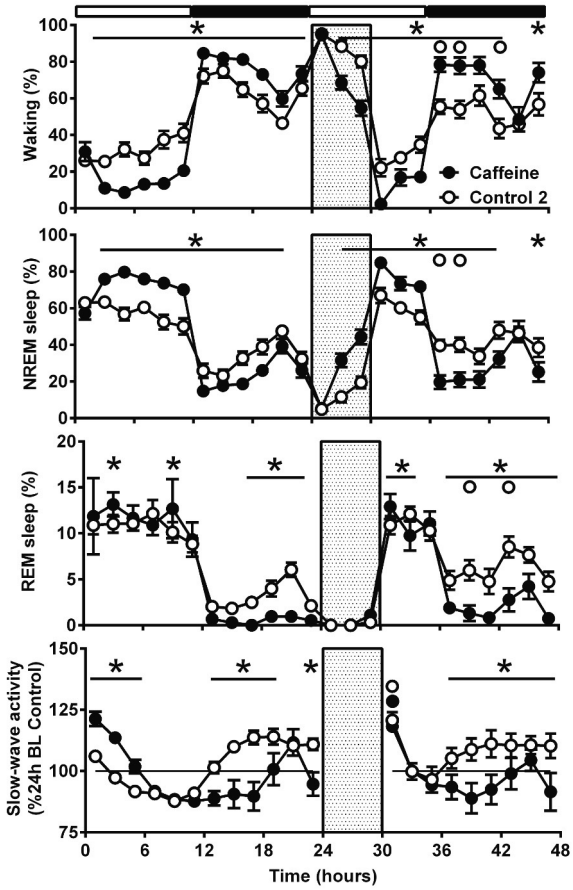

Figure 3.5: Time course of sleep-wake states and electroencephalogram (EEG) slow-wave-activity (SWA) in non-rapid eye movement (NREM) sleep, for 24 h baseline, six-hour sleep deprivation (SD, hatched area) and 18 h recovery for the second control group (n=11) and the chronic caffeine group (n=7). Curves connect two-hour values (mean±standard error of the mean (SEM)) of waking, NREM sleep, rapid eye movement (REM) sleep and EEG SWA. The black and white bars indicate the light–dark cycle. Asterisks and asterisks with lines indicate significant differences between the two groups. Significant effects of SD are indicated by open (control) and closed (chronic caffeine) circles ($p<0.05$, Bonferroni multiple comparisons test after three-way analysis of variance (ANOVA), factors 'treatment'×'time of day'×'day' with significant interactions 'treatment'×'time of day' for waking, NREM and REM sleep and 'treatment'×'day' for EEG SWA).

Discussion

Caffeine is widely used as a stimulant in order to increase waking and alertness. Validating the common conjecture that caffeine promotes arousal, the present study shows that acute intake of caffeine increases waking during the active phase. In accordance

with previous studies, sleep was found to be less deep during the following rest phase, however, without consequences for the amount of sleep. Remarkably, chronic caffeine consumption changed this picture dramatically, with increased and deeper sleep during the main sleep period.

Acute effects

After replacing the normal drinking water with caffeinated water, the amount of waking in the dark period immediately increased, at the expense of NREM sleep of which the episodes were shorter. Although the total amount of REM sleep was not significantly affected, the number of REM sleep episodes was reduced. The data indicate that, during the dark (active) phase, NREM sleep consolidation was reduced and the animals switched more often to waking. In contrast, during the following light phase, caffeine did not induce any alterations in the amount of the different sleep-wake states. Caffeine is known to increase waking and decrease sleep when administered acutely [12, 15, 16, 17, 18, 19, 20, 21, 22, 23, 30, 32, 33], and this is also our main finding in the acute condition.
The acute effects of caffeine on the waking EEG were not very pronounced and were limited to frequency bins in the theta range, indicating increased activity, exploratory behaviour and alertness [40, 41, 42, 43]. In both the NREM and REM sleep spectra, differences were evident in a few bins in the slow-wave range and in the theta range in NREM sleep. Subsequent analysis of the time course of SWA in NREM sleep showed a clear effect of acute caffeine, particularly in the dark period, in which SWA was reduced significantly. Also, at the end of the light period, SWA was lower compared with the control condition. These data are in line with previous EEG SWA changes after acute application of caffeine in rodents via i.p. injection [12, 31] and humans via oral application [19, 20, 21, 22, 23].

Chronic effects

In the chronic condition, similar effects to those in the acute caffeine condition were found during the dark period, with increased waking and decreased NREM and REM sleep. However, during the light period additional changes were found. Compared with control and acute caffeine, waking was decreased and NREM sleep was increased in the light period. This was mainly caused by a significant increase in NREM sleep episode duration, indicating that NREM sleep consolidation in the light period was increased, in comparison with both the acute and control conditions. This is a remarkable finding, considering that caffeine is generally known to decrease NREM sleep consolidation and disturb sleep [4, 5]. As a consequence, the 24-hour amplitude of the daily modulation of sleep and waking was further increased in the chronic condition. The rest-activity data obtained in the weeks after the sleep recording (Figure 1), where caffeinated water was replaced by normal water in the second week, suggest that the animals return to normal behavioural activity and sleep after caffeine withdrawal. After return to normal drinking water animals are less active during the dark and more active during the light period, compared with the period when caffeinated water was consumed.
The EEG power density spectra also showed alterations after chronic caffeine treatment.

During waking, the activity in the slow-wave range was markedly reduced and theta activity was enhanced. This is in accordance with the spectral changes seen in mice when provided with a running wheel [40, 43], suggesting that during waking in the chronic caffeine condition, the animals were more active. A similar decrease in the slow-wave range was found in the REM sleep spectrum consistent with a decrease found in an earlier study after acute caffeine consumption in humans [22].
In accordance, the changes in the daily distribution of sleep-wake states also showed a strong similarity to the changes occurring in animals with access to a running wheel, with increased sleep during the day and increased waking during the night, [43]. This is consistent with the finding that chronic caffeine, like wheel access [44], increases the amplitude of the circadian clock. Recently it was shown that caffeine not only modulates sleep homeostatic mechanisms, but also influences circadian clock function [26, 27, 28]. It has been shown that caffeine can increase circadian amplitude in vitro [25] and that the influence of light on the SCN is increased [27], which in turn may also lead to an increased circadian amplitude. An increase in the amplitude of the circadian clock may therefore underlie the increase in the 24-hour amplitude of the rest-activity and sleep-wake behaviour, and this can explain the increase in SWA and sleep pressure at the beginning of the light period. According to sleep homeostatic theory, increased waking in the active period will give a steeper increase in sleep pressure [6, 8]. The opposite will occur in the rest period. When sleep is increased, a steeper decrease in sleep pressure will occur, and this is what was observed in the data.
During NREM sleep, chronic caffeine induced a significant decrease in frequencies between 3-20 Hz. Similar to the acute condition, NREM sleep SWA was attenuated for a large part of the dark period compared with control activity. In contrast, during the first part of the light period SWA was enhanced above control levels, and reached similar levels to those of control only shortly before dark onset. The increased SWA at the beginning of the light period suggests that sleep pressure was increased compared with control pressure. This notion is supported by the finding that NREM sleep duration was also increased during this period. To investigate whether sleep pressure was indeed increased during this part of the light period, a six-hour sleep deprivation was performed. Notably, keeping the animals awake was challenging during chronic caffeine intake, compared with the control condition. This is striking since it has been shown in several studies that animals acutely treated with caffeine will stay awake for several hours [12, 18, 30] and are very easy to keep awake in sleep deprivation protocols [12] and this is the experience of several of the authors of the current study. The results of the sleep deprivation experiment confirm our notion that in the chronic caffeine condition sleep pressure is increased in the first half of the light period, compared with control pressure.
The normalization of 24-h waking in the chronic condition could be caused by a reduction of sensitivity or an increase in tolerance to caffeine which was previously observed in humans [33], cats [32] and rodents [45, 46, 47]. However, the extent of tolerance development in humans is still controversial and not clearly established [5]. The increase at the beginning of the light period in sleep and SWA in the chronic condition suggests that the sleep and slow-wave suppressing effect of caffeine is attenuated compared with the acute condition, supporting the notion that these animals are less sensitive to caffeine, or that caffeine is cleared from the system faster after they stop caffeine intake at the end of the dark period. The latter would result in larger differences in caffeine availability in

the system over the day, with less caffeine available in the light period and more in the dark. Also changes in adenosine levels or adenosine receptors could contribute to this; it has been shown that chronic administration of caffeine increases the number of adenosine receptors in the rat and mouse [48, 49]. In addition, adenosine levels were shown to be increased in rats under chronic caffeine conditions [50]. When consumption of caffeinated water is reduced, at the onset of the rest phase of the mice, all these factors may enable the physiological expression of the increase in adenosine in the system, resulting in increased sleep and SWA at the start of the light period.

The question remains as to how the data can be translated to humans. Sleep regulatory mechanisms and circadian clock functioning are similar between mice and humans [6, 7, 51]. However, humans are monophasic sleepers and active in the day, whereas mice are polyphasic and night-active. The timing of caffeine consumption relative to the rest-activity behaviour and the main sleep period is similar between humans and the mice in our experiment. Therefore, similar effects may occur in humans. On the other hand, humans have the possibility of changing to an alternative beverage when adverse effects of caffeine on behaviour or sleep occur, as opposed to the mice in our study, and this may reduce the effect of chronic caffeine consumption in those individuals.

The results in our mice show that habitual caffeine consumption has effects on sleep and sleep regulation, contrasting the effects of acute consumption of caffeine. These findings are important for the interpretation and prediction of the effects of caffeine consumption on sleep and alertness in the general population and may be useful for the therapeutic enhancement of circadian amplitude and improving sleep-wake cycles.

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Chapter 4

Sleep and dim-light-at-night duration periods

Sleep network deterioration as a function of dim-light-at-night exposure duration in a mouse model

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To be submitted

Abstract

Artificial light has a widespread use, offering several positive effects on society. However, recent research associates nocturnal light exposure with deterioration of health and well-being. One possible aspect of these disadvantageous effects includes altered circadian timing and sleep disturbances. In the present study, C57BL/6J mice were exposed to either a 12:12 h light:dark cycle (75lux:0lux) or a 12:12h light:dim-light-at-night cycle (DLAN, 75lux:5lux) and sleep electroencephalogram (EEG) and the electromyogram were recorded after one day, one week, one month, and three months of exposure. In addition to baseline 24-h recordings, a 6-h sleep deprivation was performed. We found a gradual delay of the daily 24-h vigilance state rhythms with increasing DLAN exposure duration. Although sleep was already affected following the first day of DLAN exposure, and the response to sleep deprivation was reduced in all DLAN duration periods, the most severe effects on sleep and the sleep EEG were found following three months of chronic DLAN exposure. Particularly, in all vigilance states prominent frequencies in the EEG showed a general attenuation. Additionally, by conducting detrended fluctuation analysis on the locomotor activity data, a significantly less healthy rest-activity pattern was found following three months of continuous DLAN exposure. Taking into account the behavioral, sleep and the sleep EEG parameters, the data suggest that chronic DLAN exposure impacts the sleep regulatory system and brain integrity, diminishing its adaptability and reactivity.

Keywords: sleep, dim-light-at-night (DLAN), electroencephalogram, detrended-fluctuation-analysis, sleep deprivation

Introduction

Artificial light has a widespread use in modern society. In addition to its benefits, 'light pollution' is an increasing impediment in society, i.e. light levels in the evening or night exceed natural light levels [1, 2]. Light exposure at night has been associated with various health disruptions including metabolic and immunological disturbances, as well as altered circadian timing [3]. The latter could evoke a multitude of downstream effects that consequently impacts sleep [4, 5, 6].
Sleep is coordinated by a circadian process, which is regulated by the biological clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus, and a sleep homeostatic process, which is dependent on prior waking and sleep duration [7, 8]. In mammals, the homeostatic sleep process is considered to be reflected in the NREM sleep electroencephalographic (EEG) slow-wave activity (SWA, EEG power density between 0.75-4.0 Hz) [7, 8]. Sleep deprivation has been experimentally used to test the sleep homeostatic process and investigate sleep characteristics under elevated sleep pressure conditions [8]. Whole night bedside light of 40 lux was demonstrated to have detrimental effects on sleep quality in humans including frequent arousals, decreased slow-wave sleep and reduction of SWA and activity in the spindle frequency bands during NREM sleep and theta frequency bands during REM sleep [9]. Dim-light-at-night (DLAN, 5 or 10 lux) was shown to increase waking after sleep onset, NREM sleep stage 1 and REM sleep, whereas NREM sleep stage 2 was decreased in human subjects [10]. DLAN of 5 lux was shown to disrupt molecular circadian rhythms and increase body weight in mice [11], disturb the daily sleep-wake cycle in the rat [12], and modify the expression of daily rhythms and behavioral patterns in the grey mouse lemur [13]. However, a recent study in mice showed an absence of effects of DLAN on timing as well as quality of sleep in mice [14] and, hence, more data are needed before a consensus can be reached on the effect of DLAN on sleep.
In the present study, we investigated the effect of exposure to different DLAN duration periods, i.e. one day, one week, one month and three months, on rest-activity behavior, sleep architecture, and sleep EEG parameters in C57BL/6J mice. Sleep architecture under DLAN exposure was characterized by a gradual delay of vigilance state rhythms in the course of prolonged exposure. Following three months of DLAN exposure, SWA and its 24-h rhythm was considerably attenuated, and EEG spectral alterations emerged in all vigilance states. Detrended-fluctuation analysis on the locomotor activity data [15] indicated overall a less healthy physiology. Our data suggest that not only behavioral rhythmicity and sleep, but also brain integrity and consequently general health are compromised by chronic DLAN exposure.

Materials and methods

Animals

Young adult male C57BL/6JOlaHSD mice (n=14) (Harlan, Horst, the Netherlands) were used for this study. A group of mice (n=5) was exposed to DLAN for one month in total. A second group (n=9) was exposed to DLAN for continuous three months. The age of all mice at the time of the recordings was six months. The animals were individually housed

under controlled conditions [12:12 h light:dark cycle (75lux:0lux) or 12:12 h light:DLAN (75lux:5lux); lights on at 10:00] with food and water available ad libitum in a temperature controlled room (21-22°C).
All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Centre (the Netherlands) and were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Surgeries

Under deep anesthesia (Ketamine 100 mg/kg; Xylazine 10 mg/kg; Atropine 1 mg/kg), EEG recording screws (placed above the somatosensory cortex and cerebellum) and electromyogram (EMG) electrodes (placed on the neck muscle) (Plastics One) were implanted as described previously [16, 17, 18, 19]. The wire branches of all electrodes were set in a plastic pedestal (Plastics One, Roanoke, VA) and fixed to the skull with dental cement. The mice were allowed to recover for 7-10 days.

Light schedules and behavioral recordings

Mice were kept on a 12:12 h Light:Dark schedule. At the age of five-six months, mice (n=5) were implanted with EEG and EMG electrodes, as aforementioned. In order to investigate the effect of DLAN on behavior, vigilance states and the EEG, sleep was recorded after one control day of sleep-wake recordings in light/dark conditions (Light: 75 lux, Dark: 0 lux) (control LD), after one day (1d DLAN), one week (1w DLAN) and one month (1m DLAN) exposure to 12:12 h Light:DLAN (75:5 lux) (Fig. 1a). An additional group of mice (n=9) was exposed for 12 weeks to 12:12h Light:DLAN (3m DLAN) starting at the age of three months. Subsequently, EEG and EMG electrodes were implanted and one week later the animals were recorded (Fig. 1a). At the time of the recordings the age of all mice was approximately six months. Locomotor activity for the control light-dark, the one-month and three-month conditions was monitored by passive infrared detectors (PIR, Actimetrics software, Wilmette, Il, USA) prior to the EEG/EMG recordings. For these conditions, the rhythm period and strength was determined by F-Periodogram analysis, as described earlier [12, 20].

Assessment of scale invariance using Detrended Fluctuation Analysis (DFA)

To assess the scale-invariant patterns in behavior for the mice under different conditions (control LD, 1m and 3m DLAN), we use DFA in order to calculate the correlations present in the fluctuations in behavioral activity patterns at different timescales [21, 22]. In short, a DFA calculates an averaged fluctuation value F(n) for a certain window size n. The window sizes are increased in a logarithmic manner, and for each window size a fluctuation value is determined. Then the fluctuation values F(n) are plotted against window sizes n on a log-log scale. A straight line means that there is scale-invariance in the data in the range of window sizes for which the straight line holds. The angle α can be calculated for each stretch of straight line. This value α is the scaling exponent for the range of time scales indicated by the stretch of straight line and it signifies the correlation properties in the signal. The same 15-day range that was used in analyzing

the rest-activity data for period and rhythm strength was also used to perform the DFA analysis. The circadian range for DFA analysis is from 3h up to 24h [22]. As our data above 8h are not statistically reliable due to restriction of data length [23], we used the range from 3-8 h to determine α as the circadian range.

EEG recordings

The EEG and EMG were recorded with a portable recording system (PS 1 system, Institute of Pharmacology and Toxicology, Zurich, Switzerland) as previously described [16, 17, 18, 19, 24]. Before each recording, a calibration signal was recorded on the EEG and EMG channels. Both signals were amplified, conditioned by analogue filters and sampled at 512 Hz. The signals were filtered through a digital finite impulse response filter and stored with a resolution of 128 Hz. EEG power spectra were computed for 4-s epochs by a FFT routine within the frequency range of 0.25-25.0 Hz. To record the EEG and EMG, animals were placed into experimental chambers and connected through a flexible cable and a counterbalanced swivel system to the recording setup. Conditions in the experimental chamber were similar to the home cage, including light conditions, food and water availability. Before starting each experiment, animals were allowed to adjust to the experimental conditions for a week. Subsequently, a 24-h light-dark period was recorded (control LD), starting at lights on, followed by 12-h light and 12-h DLAN (acute DLAN, 1d DLAN) (schematic overview of experimental design in Figure 1a). For the four experimental conditions, namely 1d DLAN, 1w DLAN, 1m DLAN and 3m DLAN, first a baseline day (BL, 12L:12DLAN) was recorded, followed by a second day in the same light/DLAN schedule, at the start of which six hours of sleep deprivation were conducted by gentle handling [16, 17, 18, 19, 25]. EEG and EMG were recorded continuously during BL and sleep deprivation, as well as for 18 hours of recovery period.

Data analysis and statistics

Three vigilance states (Waking, NREM sleep and REM sleep) were scored offline in 4s epochs by visual inspection of the EEG and EMG signals as well as EEG power density in the slow-wave range, as described previously [16, 17, 18, 19, 25]. For each epoch, the EEG power density in the delta (0.75-4.0 Hz) and theta band (6.25-9.0 Hz) and the integrated EMG value were graphically displayed on a PC monitor to enable scoring of the different vigilance states. The vigilance states were expressed as a percentage of artifact-free recording time. Epochs with artifacts were excluded from the subsequent data analysis of the power spectra, but vigilance states could always be determined. To test whether DLAN altered the daily vigilance state distribution a two-way ANOVA was performed (factors 'treatment' and 'time of day') on 6-h and 12-h mean values of vigilance states. In addition, a two-way ANOVA (factors 'treatment' and 'time of day') was performed in order to detect differences in the baseline (BL) time course of 2-h vigilance states values and EEG power density spectra in NREM sleep, between the different DLAN conditions and the control LD. Three-way ANOVAs were performed to investigate potential sleep alterations across different DLAN duration periods, across BL and after sleep deprivation, on 2-h and 12-h mean vigilance states values and EEG power density values in NREM sleep (factors 'treatment', 'Light-Dark', 'time of day', 'day'). Note that the 6-h value after sleep deprivation is compared to the corresponding 6-h value

of BL. A two-way ANOVA was performed on the 24-h EEG power density values of the three vigilance states with main factors 'treatment' and 'EEG frequency bins'. One-way ANOVAs (factor 'treatment', 'period', 'strength') were performed to test potential differences in 24-h vigilance state amount and in rest-activity data across all groups. When appropriate, paired and unpaired post-hoc Bonferroni-corrected student's t-tests were applied to determine the effects of treatment and sleep deprivation.

Results

Rest-activity behavioral data and DFA analysis

An overview of the experimental design is shown in Figure 1a (see more details in *Materials and Methods*). Examples of representative rest-activity behavior from animals exposed to control LD, one and three months DLAN conditions are shown in Figure 1b (15 days). F-periodogram analysis showed that the strength of the behavioral rhythm was substantially decreased following three months DLAN exposure as compared to control LD, and intermediate results were obtained following one month DLAN exposure, both one and three month DLAN groups being significantly different from control LD (Mean $\pm$ SD, Control LD: 0.21 $\pm$ 0.02, 1m DLAN: 0.14 $\pm$ 0.049, 3m DLAN: 0.11 $\pm$ 0.03, One-way ANOVA, factor 'period': $p<0.0001$, post-hoc Bonferroni multiple comparisons correction t-tests between groups).
Applying DFA on the behavioral data (more details in Materials and Methods), we found the scaling component α to be significantly lower in animals exposed to both one and three months DLAN compared to control LD (One-way ANOVA, factor 'group' $p=0.023$, post-hoc Bonferroni multiple comparisons correction t-tests between groups) (Fig. 2).

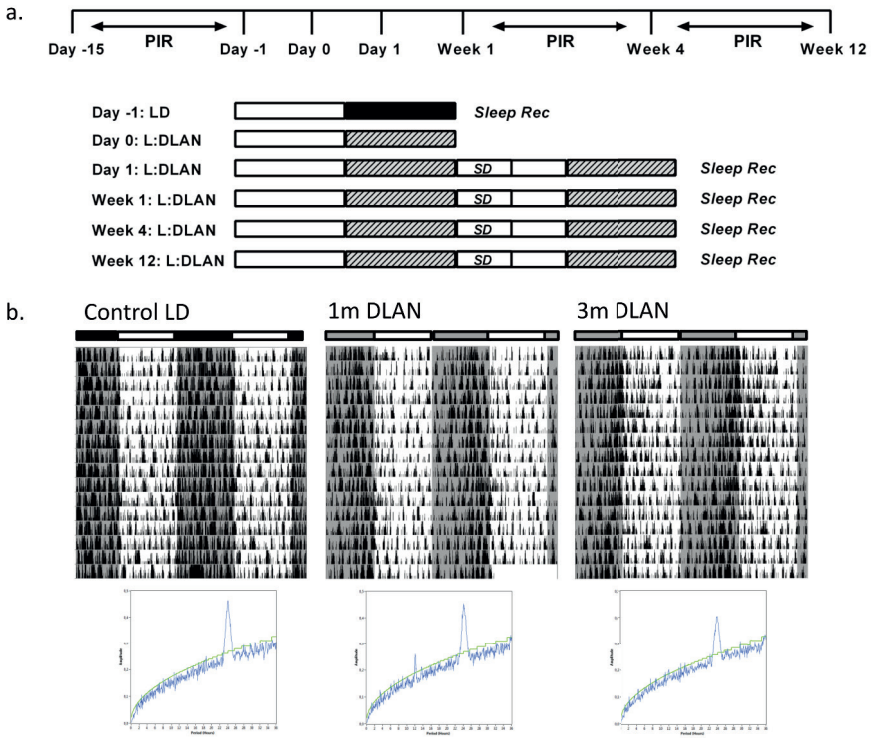

Figure 4.1: a. Schematic overview of the experimental design. Sleep was recorded during the control day (Day -1) in 12:12h Light (75 lux):Dark (0 lux) (LD) (n=5). After one night of 12-h dim light, one week, one month (n=5) and three months (n=9) of continuous exposure to 12:12h Light:Dim-light-at-night (DLAN: 5 lux), sleep recordings were repeated consisting of a baseline 24-h day followed by 6-h sleep deprivation and 18-h recovery period. Passive infrared detectors (PIR) were inserted in the cages for behavioral recordings during 15 days of control LD condition, one and three months of continuous exposure to 12:12h Light:DLAN.
b. Representative double-plotted actograms of 3 mice exposed to control light-dark (LD, 75:0lux), one month and three months 12:12h light:dim-light-at-night (DLAN, 75:5lux) with the corresponding F-periodogram analysis (Mean $\pm$ SD: *Circadian period*, Control LD: 24.0h $\pm$ 0.0, 1m DLAN: 24.4h $\pm$ 0.35, 3m DLAN: 24.08h $\pm$ 0.21, more details in the text).

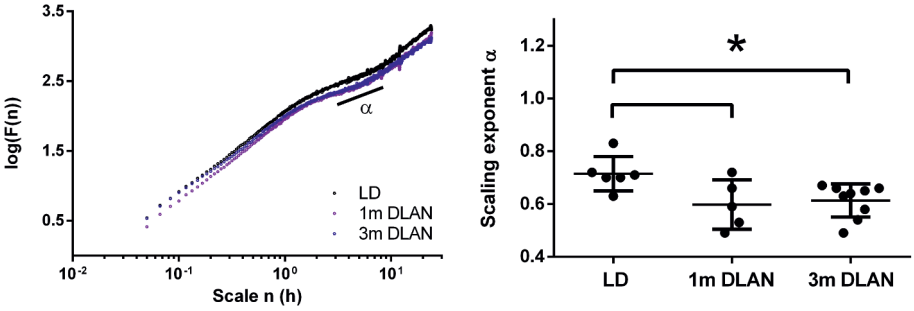

Figure 4.2: Scale-invariant correlation of behavioral activity fluctuations based on 15 days of behavioral data. Representative examples of three mice from the three different experimental groups (left panel). The scale-invariant component alpha at timescale from 3-8h (right panel) is plotted (mean ± SD) for the control light-dark group (LD), the 1 month and 3 months dim-light-at-night (DLAN) groups. Asterisks indicate significant differences between the groups ($p<0.05$, two-tailed unpaired t-tests).

Sleep architecture

In the course of DLAN exposure, waking increased and NREM sleep decreased during the light period (L1). This effect was significant after 3 months DLAN exposure, compared to control LD (Fig. 3) (post-hoc two-tailed unpaired t-tests with Bonferroni multiple comparisons correction, *Supplementary Material* Table S1). No significant changes were evident in the 12-h dark/dim light period (D1) and in 24-h vigilance state values among groups ($p>0.05$). The day-night amplitude was found to be decreased in 1w, 1m and 3m DLAN groups compared to LD and 1d DLAN groups, although this decrease was not significant (*Supplementary material*, Fig. S1). In the 6-h of the light period after sleep deprivation (L2), mice exposed to 3 months DLAN showed more waking and less NREM sleep compared to mice exposed to 1 day, 1 week and 1 month of DLAN, whereas no differences were found in the following dim light period (D2) (Fig. 3) (post-hoc two-tailed unpaired t-tests with Bonferroni multiple comparisons correction, *Supplementary Material* Table S1). Compared to BL, sleep deprivation increased NREM sleep in the light period and decreased waking in both periods (light and dim light: L2, D2) in the 1 week DLAN group and increased REM sleep in the dim light period in the 1 week, 1 month and 3 months DLAN groups (post-hoc two-tailed paired t-tests between BL and after sleep deprivation time, *Supplementary Material* Table S1).

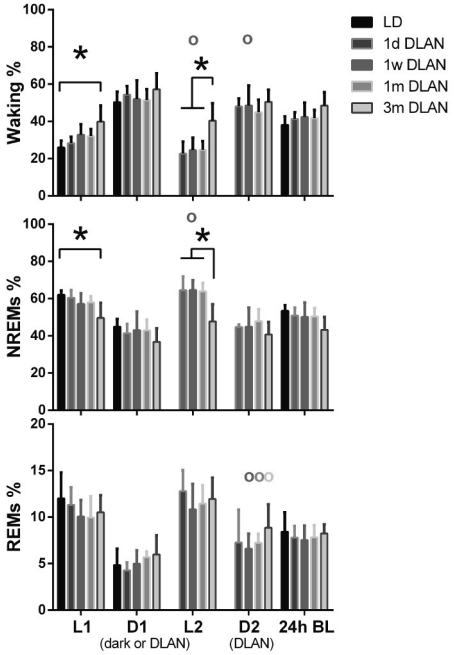

Figure 4.3: Distribution of each behavioral state (Waking, NREM and REM sleep) during the baseline day (BL) and after sleep deprivation. Bar plots represent mean (± SD) values [L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after sleep deprivation, for light and dark/dim light periods during the 48-h recordings respectively] and 24-h values of baseline recordings (24-h BL) for Waking, NREM and REM sleep for control (LD), one day, one week, one month (n=5) and three months (n=9) dim-light-at-night (DLAN) conditions. Asterisks indicate significant differences between the groups and circles indicate significant differences between recovery and BL day (post-hoc unpaired and paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'treatment', 'Light-Dark', 'day').

Additionally, vigilance state distribution over the 24-h BL day was changed in the course of DLAN exposure, showing a gradual delay in the waking peak in the dim light period after 1 day, 1 week and 1 month of DLAN exposure compared to control dark (Fig. 4) (post-hoc two-tailed unpaired t-tests with Bonferroni multiple comparisons correction, *Supplementary Material* Table S1). In the course of DLAN exposure, up to 1 month, the time in the dark/dim light period of the waking peak was positively correlated with the prior duration of DLAN exposure ($R^2 = 0.70$) (Fig. S2). After 3 months DLAN exposure, mice showed a decrease in NREM sleep during the light period, while waking and NREM sleep levels did not differ from control dark period in the first half of the dim light period and no clear waking peak was evident in the dim light period.

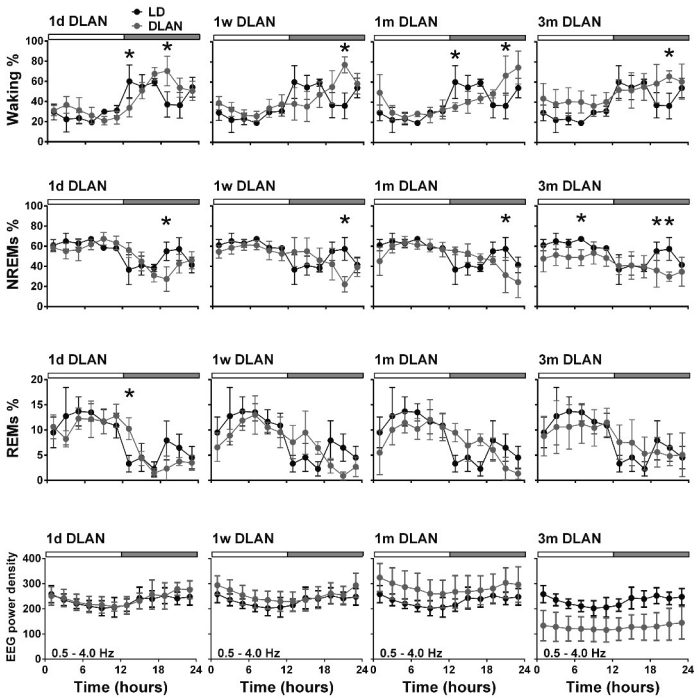

Figure 4.4: Time course of vigilance states and EEG power density in NREM sleep for frequencies 0.5-4.0 Hz (slow-wave-activity), during 24-h baseline for control light:dark (LD), one day, one week, one month (n=5) and three months (n=9) dim-light-at-night (DLAN) conditions. Curves connect 2-h mean (± SD) values of Waking, NREM and REM sleep as well as EEG power density in NREM sleep. The black curves signify LD conditions, while the grey curves DLAN conditions. The control day is plotted in each condition for easy comparison. The white and grey bars above each graph indicate the light-dark/dim light cycle (rest-active period respectively). Black asterisks at the top of each graph represent significant differences between the control LD and the different DLAN duration period conditions across the 24-h period (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVAs, main effects 'treatment', 'time of day'). Significant different EEG power levels from the control LD condition were obtained in the 1-month and 3-month DLAN groups, and 1 month and 3 month DLAN groups differed significantly from all other groups.

Throughout the day-night cycle, changes seemed to prevail in the first and second half of the dark period, whereas no large alterations were found in the light period. To investigate this further, we calculated the 6h values in the dark/dim light period. Effects that reflect the delay in waking were found in the first and second half of the dark/dim light period (D1.1 and D1.2) (Fig. S3) (post-hoc two-tailed unpaired t-tests with Bonferroni multiple comparisons correction, *Supplementary Material* Table S1). In particular, in the first 6h of the dim light period, waking was significantly decreased after 1 week and 1 month DLAN exposure, whereas REM sleep was increased after 1 month DLAN compared to control. In the second 6h of the dim light period, waking significantly increased and NREM sleep decreased after 1 week, 1 month and 3 months DLAN exposure, com-

pared to the control dark period.

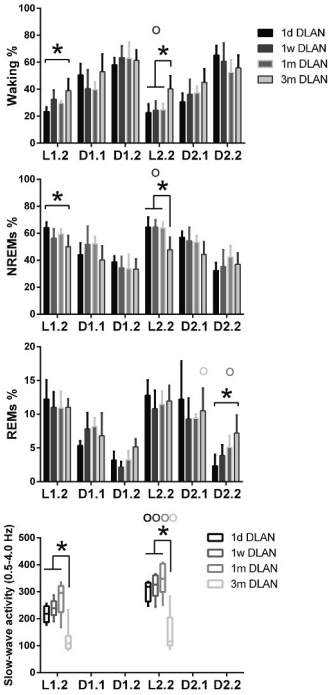

Figure 4.5: Distribution of each behavioral state (Waking, NREM and REM sleep) and slow-wave activity in NREM sleep (SWA, EEG power between 0.5-4 Hz) during baseline (BL) and during corresponding time intervals after sleep deprivation. Bar plots represent mean (± SD) 6-h values for light and dark/dim light periods and boxplots of SWA the first 2-h after sleep deprivation with the corresponding time in BL for control (LD), one day, one week, one month (n=5) and three months (n=9) dim-light-at-night (DLAN) conditions. L1.2, D1.1, D1.2, L2.2, D2.1, D2.2 correspond to 6-h values for light and dark/dim light periods during BL and following sleep deprivation. Asterisks indicate significant differences between the groups and circles indicate significant differences between recovery and BL day (post-hoc unpaired and paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'treatment', 'time of day', 'day').

Across increasing DLAN duration periods, significant differences were found between 1 day and 1 week and 1 month groups in the 2-h distribution of vigilance states (*Supplementary Material*, Fig. S4). These consisted of more waking and less NREM and REM sleep at the beginning of the dim light period in the 1d DLAN group compared to the 1m DLAN, contrary to the end of the dim light period in which more NREM sleep and less waking was found in the 1d DLAN group compared to both the 1w and 1m DLAN groups (post-hoc two-tailed unpaired t-tests, *Supplementary Material* Table S1). Following sleep deprivation, the differences in the vigilance states were more pronounced between the 3 months DLAN group and the shorter DLAN exposure groups in

which less NREM sleep and more waking was apparent in the 3 months DLAN group in the six hour light period after sleep deprivation (L2.2) and more REM sleep in the last six hours of the dim light period after sleep deprivation (D2.2) (Fig.5 and in more detail in the 2-h values in *Supplementary material*, Fig. S4). Compared to BL, mice exposed to short DLAN duration periods showed alterations in the amount of waking, NREM and REM sleep, while the 3 months DLAN exposure group showed no changes after sleep deprivation (*Supplementary Material*, Fig. S4).

EEG power density

The waking, NREM and REM sleep EEG power density spectra values were lower in the mice exposed to 3m DLAN, becoming significant in the slow-wave (1-4 Hz) and theta frequencies (5-9 Hz) compared to the control LD and the other DLAN conditions (Fig. 6) (post-hoc two-tailed unpaired t-tests with Bonferroni multiple comparisons correction), indicating that in each vigilance state the most prominent frequencies in the EEG showed a reduced power after 3 m DLAN exposure.
EEG power in NREM sleep between 0.5-4.0 Hz (EEG SWA) was altered across groups (Fig. 4, lowest panel) (post-hoc Bonferroni multiple comparisons correction t-tests between groups in the overall period of 24-h, *Supplementary Material* Table S1). Compared to the control LD group, 1 week and 1 month DLAN exposure showed a small and gradual increase in EEG SWA during NREM sleep. In accordance with the general decrease found in the EEG power density, the 3m DLAN group had the lowest EEG SWA levels. Significant different levels from the control LD condition were obtained in the 1-month and 3-month DLAN groups, whereas the 1 day and 1 week DLAN conditions did not differ from the LD control group. Additionally, 1 month and 3 month DLAN groups differed significantly from all other groups. These effects are also evident in the first 2-h following sleep deprivation, as well as in the corresponding 2-h period in BL (Fig.5, lower panel) (post-hoc Bonferroni multiple comparisons correction t-tests between groups in the 2-h light period, *Supplementary Material* Table S1). Sleep deprivation induced an increase in SWA in all groups (Fig. 5 and *Supplementary Material* Fig. S4) (post-hoc Bonferroni multiple comparisons correction paired t-tests between BL and after sleep deprivation time, *Supplementary Material* Table S1).

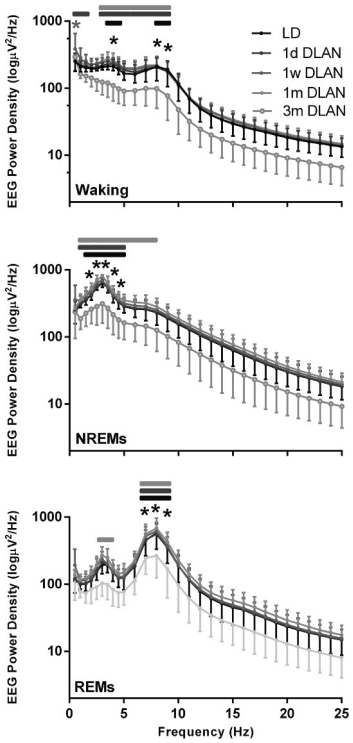

Figure 4.6: EEG power density in Waking, NREM and REM sleep for control light-dark (LD), one day, one week, one month (n=5) and three months (n=9) dim-light-at-night (DLAN) conditions during 24-h baseline (Mean ± SD) (0.5-25 Hz). Black and gray asterisks indicate significant differences between the control LD and the 3 months and 1 week DLAN groups respectively and black, dark gray and light gray lines indicate significant differences between the 3 months DLAN and the 1 day, 1 week and 1 month DLAN respectively (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'treatment', 'EEG frequency bin').

Discussion

In the present study, we found that rest-activity, vigilance state rhythms, and EEG parameters deteriorate in the course of DLAN exposure. Altered sleep parameters were evident after merely 12-h DLAN exposure and considerable consequences on locomotor activity, sleep behavior, and the EEG were found after chronic DLAN exposure. Regarding the sleep architecture, most alterations were apparent during the active period, with the waking peak being delayed following 1d to 1m DLAN conditions compared to control LD, suggesting a general delay of the main sleep and waking period occurring in the course of prolonged DLAN exposure. Moreover, after 3 months of DLAN exposure, overall increased waking and decreased NREM sleep was found during the (normally) inactive light period. Notably, SWA in NREM sleep was differentially altered in the course of

DLAN exposure. In the course of 1w and 1m DLAN, it was gradually increased compared to control LD, whereas after 3m of DLAN exposure it was greatly attenuated, below control LD levels. In parallel, a general decrease in prominent frequencies (slow-waves and/or theta) of all vigilance states was found after 3 months DLAN exposure. Regarding the behavioral analysis, DLAN exposure induced a delay in the activity onset in the dim light period and a gradual decrease in the strength of the rhythm after 1 and 3 months. Interestingly, DFA on the rest-activity data revealed a decrease in the scaling component suggesting attenuated adaptability and integrity of the circadian system. Our data show that long-term DLAN exposure can negatively impact sleep and daily rhythms influencing the overall physiology and behavior.

Sleep architecture

An important aspect of the effect of DLAN on vigilance states distribution is the delay of the daily maximum of the active waking period in the dim light period, compared to control LD dark period. This delay lasted from 2-8 hours and increased as a function of DLAN exposure from 1 day to 1 month (Fig. 4 and Fig. S2). Although the dynamics of the daily light-dark/dim amplitude were not significantly disturbed following 1d, 1w, 1m of DLAN exposure, they seem to become distorted after 3 months DLAN exposure. In this group, waking was increased during the light period. Similar to the delayed activity and waking peak, delayed temperature rhythms were noted after 2 weeks of DLAN exposure in the grey mouse lemur [13]. A recent study in Wistar rats, also showed a gradual reduction in rhythm amplitude in vigilance state distribution, starting from day 1 to day 14 of DLAN exposure [12]. Our data are in accordance with Stenvers et al study (2016), however many effects in our mice occurred at a later time point. As our light levels were very similar this suggests that longer exposure to the DLAN condition was required before effects were visible in the mice. Stenvers et al (2016) performed their study in albino rats, whereas our study was conducted in pigmented mice, which may explain this difference in duration. In general, pigmented animals are less susceptible to light compared to albino animals [26, 27]. In contrast, in a study in Swiss Webster mice, long-term DLAN exposure did not induce any alterations in sleep architecture [14]. However, in that study, in addition to an eight-week DLAN exposure, a running wheel was available in the cage. Introduction of a running wheel for mice, that can be used long-term voluntarily on a daily basis, has been shown to substantially affect sleep and the sleep EEG [28, 29]. Interestingly, we recently shown that voluntary use of a running wheel on a daily basis is able to improve sleep architecture and the sleep EEG of mice substantially, lasting for at least two weeks after removal of the wheel [19]. In the current study, we show that in a DLAN condition in animals that do not receive this cage enrichment, quality of sleep will deteriorate and the distribution of sleep will become more random, but at a slower rate than seen in albino rats.

In order to test the sleep homeostatic response after elevated sleep pressure, we conducted a 6-h sleep deprivation in all DLAN groups [16, 17, 18, 19, 25]. Sleep deprivation induced differences among DLAN groups, separating the 3m DLAN from the other groups, which showed more waking and less NREM, during the 6-h of the recovery light period (Fig. 3, 5). Although mice exposed to all DLAN durations were sleep deprived equally successful, it seems that sleep deprivation did not restore behavior during the subsequent

recovery period, as if the mice were less susceptible to elevated sleep pressure. Only mice exposed to 1w DLAN showed the normal increase in NREM sleep during the 6-h recovery light period after sleep deprivation (Fig. 3, 5). Consistent between DLAN durations was the increased REM sleep, particularly in the first part of the active period after sleep deprivation in the 1w to 3m DLAN conditions.

Rest-activity behavior and fractal patterns

In order to decipher the impact of DLAN exposure on daily rhythms, we conducted additional analysis on the rest-activity data. DFA has been used in many studies to quantify correlations in physiological time series [30]. With DFA information enclosed in physiological data reveals the potential effects of network fluctuations at different timescales. As demonstrated by a large body of literature [31, 32], scale-invariant correlations with $\alpha \sim 1$ are associated with healthy physiology, which is not too responsive to external perturbations, but flexible enough to adapt to these external perturbations (adaptive, but not reactive). In our study, DFA revealed an α close to 1 in the control LD condition and α decreased as a function of DLAN duration. Most healthy physiological systems show scaling components around 1, whereas deviations from 1 are associated with disease or aging [15, 22]. The pattern of alterations as indicated by the fractal view of physiology likely describes underlying mechanisms of network-wide responsiveness and adaptability.
Exposure to DLAN is likely to influence functioning of the circadian clock in the SCN. The SCN appears to have regulatory functions at multiple time scales, which exceed the conception of the traditional circadian time with values around 24 hour [33]. We are showing and using the 3-8 h timescale which is known to be influenced by the circadian clock [34]. In the course of prolonged exposure to DLAN, mice in our study exhibit a gradual decrease in their α values. This shows that DLAN, particularly when it is chronic, can attenuate daily fluctuations corresponding to healthy physiology. In accordance, following a long-term DLAN protocol of four weeks, it was shown that Swiss Webster mice increased their percentage of food consumed especially during the light period, and revealed disrupted molecular circadian rhythms during the active period [11]. Our data suggest that the complex network of the brain that regulates circadian behavior is less adaptable to perturbations and its integrity is negatively affected by DLAN [30].

EEG power density in waking, NREM and REM sleep and slow-wave activity (SWA) in NREM sleep

The EEG is a window on the brain, and alterations in the EEG can be interpreted in the context of neuronal network integrity and adaptability [17, 35]. Spectral analysis of the EEG revealed that in waking, NREM and REM sleep, mice exposed to 3 months DLAN had significant differences with control LD as well as the 1d, 1w, and 1m of DLAN. Particularly, mice that were exposed to 3m DLAN showed a general decrease in slow delta activity in NREM sleep and theta frequencies activity in REM sleep and waking compared to all the other experimental groups. SWA levels in NREM sleep showed similar patterns, being slightly increased across 48-h in short- and mid-term DLAN exposure up to 1 month, however, demonstrating a great general decrease after 3 months of DLAN exposure. In accordance with the attenuated rhythmicity in rest-activity after 3 months

DLAN exposure, the spectral alterations suggest a reduced quality of brain cortical activity in all three vigilance states. The subtle decrease in slow-wave activity in the waking spectra after 3 months DLAN exposure suggests that these mice are less drowsy [36, 37]. However, the concomitant more robust decrease in waking theta, indicates that these animals are also less active and alert [36, 37]. These are two contradicting findings, and therefore these data suggest that brain integrity in general may be affected by long-term DLAN exposure and that this is reflected in the EEG.
In humans, nocturnal light exposure of 40 lux had similar effects on the sleep EEG, where slow, delta and spindle frequency activity in NREM sleep as well as theta activity in REM sleep was decreased [9]. In addition, rats exposed to 14 days of DLAN, showed decreased SWA during the light period, increased SWA at the end of the active period and a general decrease in EEG activity between 16-19 Hz during NREM sleep [12]. In contrast, Swiss Webster mice showed no alteration in SWA after eight weeks of DLAN exposure compared to control LD. However, in the latter study the daily modulation in SWA was also lacking in the control LD group [14]. Although the increase in NREM sleep after sleep deprivation was attenuated, after DLAN exposure, all DLAN groups showed an increase in SWA levels during the first 2h after sleep deprivation. This shows that, although the amount of NREM sleep did not always increase, part of the homeostatic sleep response, expressed in NREM sleep SWA, was still functioning [7, 8].
As aforementioned, the deterioration of all prominent EEG frequencies across the three different vigilance states could imply attenuation in the EEG generation caused by alteration in the neuronal network underneath. Rhythmic events within the delta frequency range are intrinsically generated in thalamic neurons [38]. However, this activity cannot reach the cortex to be reflected at the macroscopic EEG level, unless thalamic neurons are synchronized [38]. Thalamo-cortical projections underlie the performance of specific functional tasks during alert as well as sleep states. Therefore, the general decrease in EEG power density in the low frequencies may be caused by a loss of thalamocortical synchronization due to prolonged DLAN exposure. Furthermore, since the cerebral cortex is not merely a passive receiver of synchronized delta potentials of thalamic origin, but these inputs are reorganized by the intrinsic properties and synaptic events in cortical circuits [38, 39], alterations following DLAN exposure may also take place at the level of the cortex.
In short, chronic DLAN exposure induces a reduction of the slope α in the DFA analysis of locomotor activity, indicating a less adaptive and potentially a more reactive system. In addition, sleep and EEG data suggest an overall altered thalamo-cortical neuronal network. When challenged with a perturbation, in this case a sleep deprivation, the response is inadequate as the increase in NREM sleep is attenuated. Therefore, the data suggest that the dynamics of the underlying sleep regulatory mechanisms are altered and that the integrity of the brain network is distorted following chronic DLAN exposure.
The benefits of electricity and electric lighting are indisputable. Nevertheless, recent research associates light exposure at night with health consequences. The first deleterious effects regarding sleep in our study are apparent after one night of DLAN exposure. Prolonged exposure to DLAN induces additional rest-activity rhythm disruptions together with further sleep and EEG quality degradation. The scale invariant component of DFA is reduced indicating unhealthy correlations under DLAN that can possibly render the organism not able to adjust to altered environmental conditions, which is confirmed by

the results of the sleep deprivation experiment. Concluding, DLAN induces a diminished integrity of the circadian, sleep, and EEG regulatory systems and probably has additional effects on neurophysiological systems, impacting sleep health as well as the underlying brain network.

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Supplementary Material

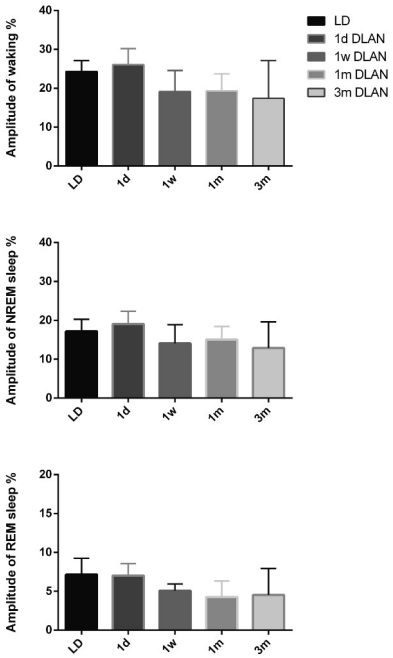

Figure 4.1: Amplitude of the daily rhythm of vigilance states (Waking, NREM and REM sleep) for control (LD), 1 day, 1 week, 1 month (n=5) and 3 months (n=9) dim-light-at-night (DLAN) conditions during 24-h baseline recordings (Mean $\pm$ SD).

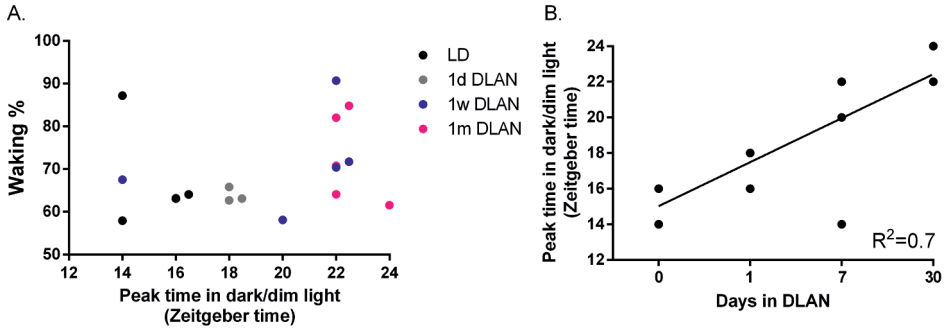

Figure 4.2: Relationship between the waking peak time during the dark/dim-light period (active period) and the amount of time spent in dim-light-at-night (DLAN). A. The amount of waking during the 2-h peak waking time for the experimental groups (control LD, 1 day DLAN, 1 week DLAN, 1 month DLAN). B. A positive correlation was found between the waking peak time and the amount of time spent in the DLAN condition from 0 days up to 30 days (n=5 for each condition) ($R^2 = 0.6999$).

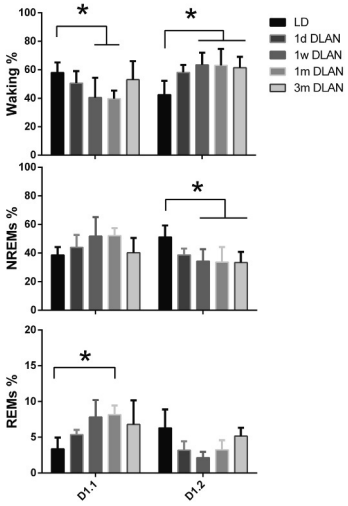

Figure 4.3: Distribution of each behavioral state (Waking, NREM and REM sleep) during the baseline dark/dim light period. Bar plots represent mean (± SD) values (D1.1, D1.2) corresponding to 6-h values for dark/dim light periods for control (LD), 1 day, 1 week, 1 month (n=5) and 3 months (n=9) dim-light-at-night (DLAN) conditions. Asterisks indicate significant differences between the groups (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, p<0.05 after significant ANOVA, main effects 'treatment', 'Light-Dark').

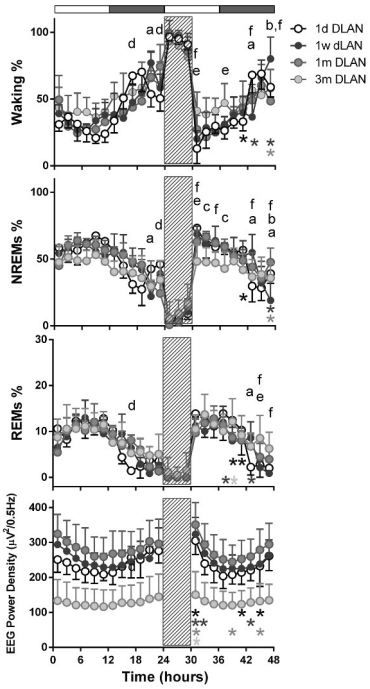

Figure 4.4: Time course of vigilance states and EEG power density in NREM sleep in 0.5-4.0 Hz (EEG SWA), for 24-h baseline (BL), 6-h sleep deprivation (hatched bar) and 18-h recovery for 1 day, 1 week, 1 month (n=5) and 3 months (n=9) dim-light-at-night (DLAN) conditions. Curves connect mean (± SD) 2-h values of Waking, NREM and REM sleep. The white and grey bars above each graph indicate the light-dim light cycle (inactive-active period respectively). Letters at the top of each graph represent significant differences between the groups (a: 1d-1w, b: 1w-1m, c: 1m-3m, d: 1d-1m, e: 1d-3m, f: 1w-3m DLAN) across the 48-h period and asterisks at the bottom of each graph significant differences between recovery and BL day for each DLAN group (post-hoc unpaired and paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVAs, main effects 'treatment', 'time of day', 'day'). Characteristic low EEG SWA levels were found in the three months DLAN group compared to all other groups.

Part 2

Aging data

Chapter 5

Sleep and EEG changes in aging

Differences in electroencephalographic non-rapid- eye move-ment sleep slow-wave characteristics between young and older mice

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Abstract

Changes in sleep pattern are typical for the normal aging process. However, aged mice show an increase in the amount of sleep, whereas humans show a decrease when aging. Mice are considered an important model in aging studies, and this divergence warrants further investigation. Recently, insights into the network dynamics of cortical activity during sleep were obtained by investigating characteristics of individual electroencephalogram (EEG) slow waves in young and elderly humans. In this study, we investigated, for the first time, the parameters of EEG slow waves, including their incidence, amplitude, duration and slopes, in young (6 months) and older (18-24 months) C57BL/6J mice during undisturbed 24h, and after a 6-h sleep deprivation (SD). As expected, older mice slept more and, in contrast to humans, absolute NREM sleep EEG slow-wave activity (SWA, spectral power density between 0.5-4 Hz) was higher in the older mice, as compared to the young controls. Furthermore, slow waves in the older mice were characterized by increased amplitude, steeper slopes and fewer multipeak waves, indicating increased synchronization of cortical neurons in aging, opposite to what was found in humans. Our results suggest that older mice, in contrast to elderly humans, live under a high sleep pressure.

Introduction

Aging has been associated with numerous and diverse changes in sleep. In humans, these include increased sleep fragmentation, decreased total sleep time, sleep efficiency and slow-wave-sleep (SWS), and attenuated electroencephalogram (EEG) slow-wave activity (SWA, spectral power density between 0.75-4 Hz) in non-rapid eye movement (NREM) sleep [1, 2, 3, 4]. In parallel with SWA, the amplitude, slope and density of the NREM sleep slow waves was decreased in middle aged (41-60 y.o.) and older humans (50-70 y.o.) [5, 6]. In mice, aging impacts the amplitude and timing of circadian rhythms as well as sleep quality [7, 8, 9, 10, 11, 12]and waking performance [13, 14, 15]. Several studies showed that the time spent asleep over 24 hours increased with age in mice, particularly during the dark period [7, 8, 9, 10, 11, 12]. Since aged mice show increased sleep fragmentation and increased sleep propensity in their habitual waking period during the night as well as less pronounced circadian rhythms of sleep and wakefulness [7, 11], they have been considered a good model to investigate age-dependent changes in sleep physiology. In humans, changes in NREM sleep SWA and slow-wave characteristics were studied thoroughly and were found to be consistent with the decrease in the amount of sleep seen in aging [1, 2, 3, 4, 5, 6]. However, in mice the effects of age on slow-wave parameters, that could provide insight into cortical network properties, have not been studied, and it remains unknown whether EEG changes occur in parallel with global sleep-wake alterations.

Sleep is considered to be mainly regulated by two processes [16, 17]. The timing of sleep is regulated by the circadian clock which in mammals is located in the suprachiasmatic nucleus (SCN) of the hypothalamus. The depth of sleep is homeostatically regulated, as reflected in progressively increasing sleep propensity during wakefulness and its dissipation during sleep. Prolonged waking is compensated by deeper and sometimes longer subsequent sleep [16]. In mammals, the homeostatic sleep process is thought to be reflected in the NREM sleep EEG SWA16. The overall level of EEG SWA is determined mostly by the incidence, as well as amplitude of individual EEG slow waves, which show distinct changes across the sleep period [18, 19, 20]. In addition, slow-wave slopes and the number of peaks within a single wave (multipeak waves) are thought to reflect network synchronization [18, 19, 20, 21]. During early sleep, when SWA is high, there are more slow waves with large amplitude, their slopes are steep, and multipeak waves are rare [18, 19, 20]. As sleep progresses, SWA diminishes, reaching lowest levels at the end of the main sleep period [16, 22]. During that period there are fewer large-amplitude slow waves, their slopes are less steep, and multipeak waves are more frequent [18, 19, 20]. Sleep deprivation (SD) experiments have shown that SWA in NREM sleep increases as a function of prior waking duration and decreases again during subsequent recovery sleep [16]. The incidence and morphology of the slow waves were shown to follow an analogous pattern with increased incidence, amplitude and slopes and decreased multipeak waves [18, 19, 20].

Investigating the parameters of individual slow waves, such as the amplitude, duration and slopes, provides important insights into the functioning of the underlying neuronal network. It has been established, that EEG slow-wave fluctuations reflect synchronized activity of large cortical populations near the recording electrode, as well as activity originating from distant sources, influencing the activity patterns among neurons, located near

the electrode [23]. Previous studies, where EEG or local field potentials were combined with cortical neuronal activity recordings, revealed that sleep slow waves are a reflection of near-synchronous transitions, between up (active) and down (silent) state in large populations of neurons, as well as, that steeper slow-wave slopes are associated with tighter synchrony at the transitions between activity and silence [18, 24, 25, 43, 27, 28]. The observations from the aforementioned research studies provide the opportunity to study the network mechanisms, underlying age-dependent changes in cortical oscillations, by recording the NREM sleep EEG in the aging mouse model.
In this study, we investigated for the first time, the incidence and characteristics of NREM sleep slow waves, including their amplitude, duration, number of peaks within a single wave and slopes in the aging mouse model to define the cortical network characteristics. We conclude that the underlying cortical network likely shows increased synchronization in older mice (18-24 months old) compared to young controls (6 months old), resembling the effects of an increase in sleep pressure.

Materials and methods

Animals

Young (6 months old; n=9) and old (18-24 months old; n=24) male C57BL/6JOlaHsd mice (Harlan, Horst, the Netherlands) were used for this study. All mice were housed under controlled conditions (12:12 h light:dark cycle; lights on at 09:00) with food and water *ad libitum* in a temperature controlled room (23-24 °C). The animals were individually housed in cages with a running wheel three months prior to the experiment for cage enrichment and increased chances of healthy aged mice [29]. All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Center (the Netherlands) and were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Surgeries

When the animals reached the required age, they were operated under deep anesthesia (Ketamine 100 mg/kg; Xylazine 10 mg/kg; Atropine 1 mg/kg) and EEG recording screws and electromyogram (EMG) electrodes (Plastics One) were implanted as described previously [30, 31]. One EEG electrode was placed over the right hemisphere (2 mm lateral to the midline of the skull, 2 mm posterior to bregma) above the somatosensory cortex, while the other was placed above the cerebellum (midline, 2 mm caudal to the lambda) as a reference. The wire branches of all electrodes were set in a plastic pedestal (Plastics One, Roanoke, VA) which was fixed to the skull with dental cement. The mice were left to recover for at least 7-10 days.

EEG recordings

The EEG and EMG were recorded with a portable recording system (PS 1 system, Institute of Pharmacology and Toxicology, Zurich, Switzerland) as previously described [30, 31, 32]. Before each recording, a calibration signal (10 Hz sine wave 300 μV peak-to-peak) was recorded on the EEG and EMG channels. Both signals were amplified (am-

plification factor $\tilde{2}$,000), conditioned by analogue filters (high-pass filter -3 dB at 0.16 Hz) and sampled at 512 Hz. The signals were filtered through a digital finite impulse response filter (EEG low-pass filter at 30 Hz and EMG band-pass filter between 20 and 40 Hz) and stored with a resolution of 128 Hz (with 12 bit amplitude resolution). EEG power spectra were computed for 4-s epochs.
To record the EEG and EMG, animals were placed into experimental chambers and connected through a flexible cable and a counterbalanced swivel system to the recording setup. Conditions in the experimental chamber were similar to the home cage. Before starting the experiment, the animals were allowed to adjust to the experimental conditions for at least a week. Subsequently, a baseline (BL) day was recorded, starting at lights on. At the start of the second day, six hours of SD were conducted by gentle handling [30, 33]. During that period, every time the animals appeared drowsy or the EEG exhibited slow waves, the animals were mildly disturbed by noise, movement of bedding or introducing new nesting material or food into the cage. EEG and EMG were recorded continuously during SD and, subsequently, for 18 hours to investigate sleep after SD.

Data analysis and statistics

Three vigilance states (REM sleep, NREM sleep and Waking) were scored offline in 4s epochs by visual inspection of the EEG and EMG signals as well as EEG power density in the slow-wave range, as described previously [30, 31]. Epochs with artifacts were excluded from the subsequent data analysis of the power spectra, but vigilance states could always be determined. Corresponding light and dark mean values and 2-hourly values of vigilance states and EEG SWA in NREM sleep were analyzed. The distribution of waking, NREM sleep and REM sleep episode duration was analyzed according to previously published criteria [34, 35, 36]. Episodes of each vigilance state were partitioned into ten bins with exponential increased duration from 4s to >1024s. Spectral analysis was performed using a fast Fourier transform (FFT; 0.5-25 Hz, 1 Hz resolution). Wake, NREM and REM sleep EEG spectra were computed during the BL. Absolute values for the SWA were computed to increase the possibility to compare this with the routinely available absolute slow wave data in humans [2, 5, 19, 37, 38]. The decay rate in NREM sleep SWA after SD was calculated between the first and third hour, by calculating the proportion of the first 30min-value after SD to the sixth 30min-value (3rd hour) after SD. A one-way, two-way or three-way analysis of variance (ANOVA) with factors age, time of the day, day, Light-dark, duration/frequency bin, and amplitude levels, was applied to test the differences between the age groups and experimental conditions. When the ANOVA reached significance, paired and unpaired t-tests were applied when appropriate to determine the effects of SD or age. R-values were averaged after fisher-Z transformation.

Detection and Analysis of Slow Waves

Detection of individual slow waves was performed on the EEG signal after band pass filtering (0.5-4 Hz, stopband edge frequencies 0.1-10 Hz) with MATLAB filtfilt function exploiting a Chebyshev Type II filter design (MATLAB, The Math Works, Inc., Natick, MA) [39]. As a first step, slow waves were detected both as positive and negative deflections of the EEG signal between two consecutive negative (or positive respectively)

deflections relative to the zero-line separated by at least 0.1 seconds. Subsequently, for each animal, we calculated independently integrated EEG amplitude in a spindle frequency range (band pass filter: 9-13 Hz, stopband edge frequencies 7-15 Hz, Chebyshev Type II filter design, MATLAB, The Math Works, Inc., Natick, MA), and computed slow-wave triggered average of this new time series both for positive and negative slow waves. As it is well known, population 'down-states' correspond to reduced faster EEG frequencies, while spindles are often associated with an up-state both in humans and animals [40, 41, 42]. The resulting two time series were then subjected to further analysis. For the final analyses, we selected for each individual animal either positive or negative EEG slow waves, which corresponded to a suppression of corresponding amplitude of EEG signal in a spindle-frequency range. This procedure ensured that all analyses were performed not on arbitrarily selected EEG negative waves, but on EEG potentials corresponding to reduced network activity. There is evidence, that the first segment of slow waves corresponds to the down phase of the slow oscillations, whereas the second segment corresponds to the up phase [39, 40, 43, 44]. The peak-to-peak amplitude of the first and second segment of the wave, the slopes (mean first derivative of the first and second segment), and the number of peaks within a single wave were computed for each individual wave during NREM sleep. Multipeak waves were defined as waves with more than 1 peak between the beginning and end of the wave.
All slow waves were sorted into 5 amplitude percentage ranges (5 quintiles, 20 percentiles each) according to the amplitude of the peak (from zero-crossing to maximum value), as it was shown in previous studies [18, 19] and all the parameters of individual slow waves were computed for each amplitude percentage range, since, as it is reported previously, the most important determinant of power in the SWA band is the number of high-amplitude slow oscillations20. The above-mentioned analysis was performed in the 6 months old (n=9) and 18 months old (n=9) group.

Results

Vigilance states and episode duration

The aged mice showed increased NREM sleep and decreased waking, especially in the dark period (Fig. 1a) (2-way ANOVA, interaction factors 'age*time of day'; Waking: $p<0.0001$, NREM: $p<0.0001$). During undisturbed BL, REM sleep was decreased in the older animals at the end of the light period and increased during the first part of the dark period, as compared to the young animals. Also in the dark period after SD, REM sleep was increased in older mice as compared to young controls (2-way or 3-way ANOVA, interaction factors 'age*time of day'; REM sleep: $p<0.0001$ and 'age*time of day*day'; REM sleep: $p<0.0001$).
Episodes of each vigilance state were partitioned into ten bins with exponential increased duration from 4s to >1024s [34, 35, 36] (Fig. 1b). The largest differences between young and old animals were found in the dark period. During both dark periods, aged animals showed increased number of the longer NREM sleep episodes (2-way or 3-way ANOVA, interaction factors 'age*duration'; $p=0.016$, 'age*Light-dark'; $p<0.0001$, 'age*Light-dark*duration'; $p=0.053$), more short and middle duration waking episodes and a decrease in the longest waking episodes (>1024 sec) compared to young controls. In the

BL light period, older animals had fewer short waking episodes and a higher incidence of longer episodes (2-way or 3- way ANOVA, interaction factors 'age*duration'; p=0.002, 'age*Light-dark'; p<0.0001, 'age*Light-dark*duration'; p=0.001). In both light periods, older mice showed a decrease in the 128s-bin REM sleep episodes and in the dark period an increase in the number of shorter REM sleep episodes compared to the young animals (2-way or 3- way ANOVA, interaction factors 'age*duration'; p<0.0001, 'age*Light-dark'; p<0.0001, 'age*Light-dark*duration'; p=0.730) (data shown only in BL).

Absolute EEG power density and absolute SWA

To investigate whether gross alterations in sleep-wake amount and distribution are associated with specific changes in cortical EEG, we calculated the spectral distribution of absolute EEG power density in the frequencies between 0.5 and 25 Hz (Fig. 2). Aged mice had a significantly higher EEG power density in the frequencies between 2-7 Hz during NREM sleep, as compared to young controls. In waking, EEG spectra in old animals were characterized by a pronounced decrease in power density on the high end of the theta peak (9-12 Hz), whereas power density around 3 Hz was slightly higher. These observed differences were not only frequency- but also vigilance state specific, as no significant differences were found in the REM sleep EEG spectra (2-way ANOVA, interaction factors 'age*frequency bins'; Waking: p<0.0001, NREM sleep: p<0.0001, REM sleep: p=0.332).

In the old mice, higher absolute SWA in NREM sleep was observed over the entire 48h recording period (one-way ANOVA, factor 'age'; p<0.0001, (t, df)=(9.329, 46); p<0.0001) with significant differences during the entire light period and at the end of the dark period, where most sleep occurs (Fig. 1a, bottom panel). Further age differences were apparent in the dynamics of SWA after sleep deprivation. Specifically, the decay rate in NREM sleep SWA between the first and third hour after SD, was significantly slower in the old animals [young: 154.58% $\pm$ 7.55; old: 138.04% $\pm$ 3.75, unpaired t-test (t, df)=(2.164,31); p=0.038, Supplementary material, Fig. S1].

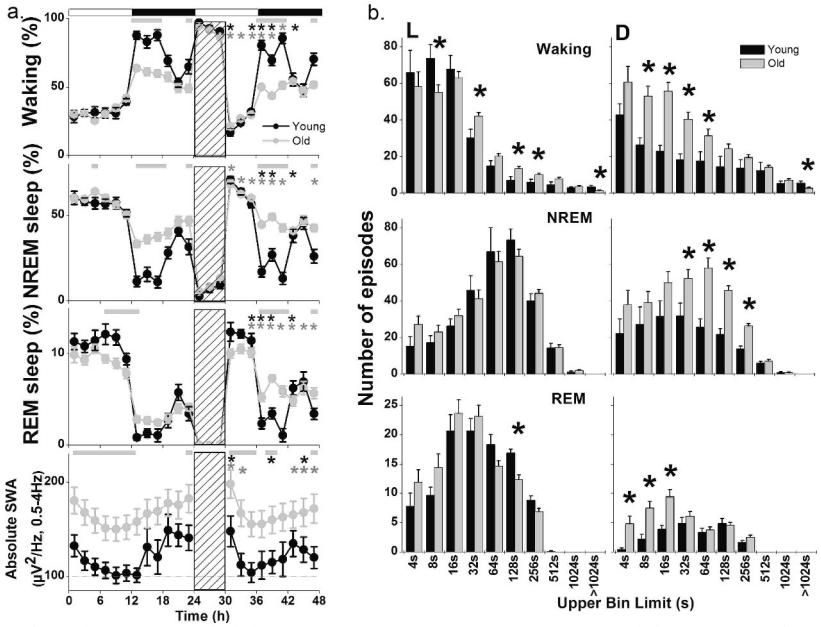

Figure 5.1: Time course of vigilance states and absolute slow-wave activity (SWA, 0.5-4Hz) in non-rapid-eye movement (NREM) sleep, and episode frequency histograms of vigilance states. a. Time course of vigilance states and absolute SWA in NREM sleep, for 24-h baseline, 6-h sleep deprivation (SD, hatched bar) and 18-h recovery for the two age groups, young (black circles, n=9) and old mice (gray circles, n=24). Curves connect 2-h values (mean ± SEM) of Waking, NREM, REM sleep and absolute SWA. The black and white bars indicate the light-dark cycle. Horizontal gray lines represent significant differences between young and old across the 48-h period and black (young) and gray (old) asterisks significant differences between recovery and baseline day (unpaired and paired t-tests, $p<0.05$ after significant 1-, 2- or 3-way ANOVA, interaction factors 'age', 'age*time of day' and 'age*time of day*day').

b. Episode frequency histograms of Waking (upper), NREM (middle) and REM sleep (lower panel) in the Light (L, left) and Dark (D, right) period during baseline for young (black bars, n=9) and old mice (gray bars, n=24) (mean values ± SEM). Episodes are partitioned into ten exponentially increased duration bins from 4 to >1024s (x axis designates the upper limit of each bin for the following bins: 0-4, 5-8, 9-16, 17-32, 33-64, 65-128, 129-256, 257-512, 513-1024, >1024). Asterisks indicate significant differences between young and old animals (unpaired t-tests, $p<0.05$ after significant 2- or 3-way ANOVA, interaction factors 'age*duration', 'age*Light-dark' and 'age*Light-dark*duration').

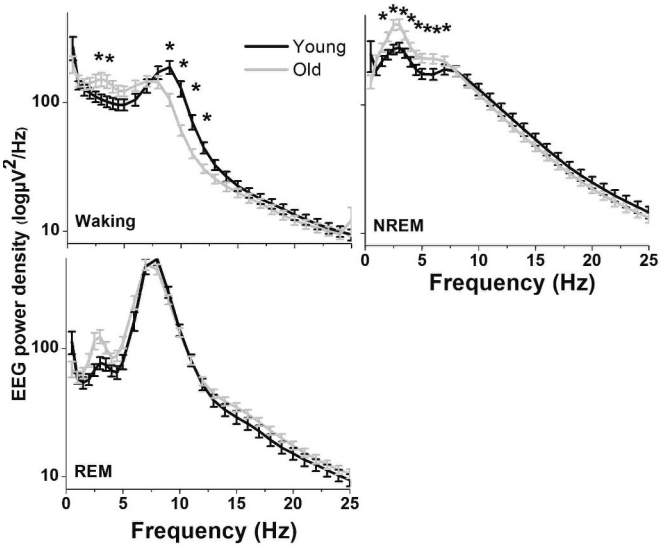

Figure 5.2: Spectral distribution of electroencephalogram (EEG) power density (mean values ± SEM) in the frequencies between 0.5 and 25 Hz for young (black, n=9) and old mice (gray, n=24) in Waking (top left), non-rapid-eye movement (NREM, top right) and REM sleep (bottom left) computed for pooled values of the 24-h baseline day. Asterisks indicate significant differences across the frequency bins between the two groups (unpaired t-tests, p<0.05 after significant 2-way ANOVA, interaction factors 'age*frequency bins').

Slow-wave morphology
Average EEG slow wave slopes and sigma activity

An example of a trace of raw EEG from a young mouse, with a slow wave followed by a spindle-event, defined as burst of activity in the range between approximately 9-13 Hz, is shown in Fig. 3a. In Figure 3b, two examples are depicted, which show the average EEG slow wave from an individual young and an old mouse, along with the corresponding rectified amplitude of the EEG signal, filtered in the sigma frequency range (9-13 Hz). Notably, a higher amplitude slow wave is observed in the older animal. In the average values, steeper absolute first and second slopes were found in the old compared to young mice, in the main sleep period [1st slope, Young: 808.01 μV/s $\pm$ 8.61; old: 1018.1 μV/s $\pm$ 8.44, unpaired t-test (t, df)=(17.429, 538); p<0.0001, 2nd slope, Young: 941.15 μV/s $\pm$ 8.82; old: 1126.8 μV/s $\pm$ 6.94, unpaired t-test (t, df)=(16.541, 509.9); p<0.0001], and increased absolute slow-wave amplitude and slopes in the old compared to the young, whereas no significant differences were found in the slow-wave duration (Fig. S2, data of the highest-amplitude slow waves shown). Furthermore, we observed a deeper trough in the average sigma activity, corresponding to the negative phase of the slow wave, in the old as compared to the young mice [Fig. 3C, unpaired t-test for the trough period (t, df)=(2.252, 52); p=0.0286].

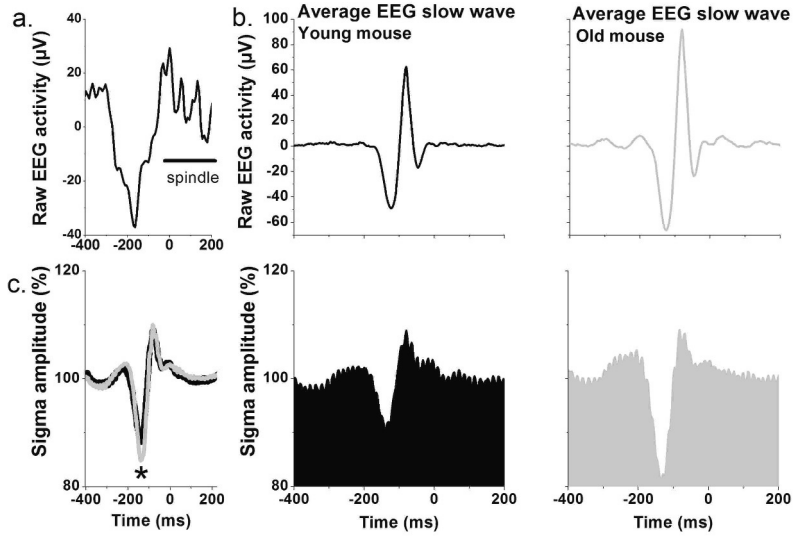

Figure 5.3: Average electroencephalogram (EEG) slow wave and Sigma amplitude. a. Representative EEG trace of a slow wave followed by a spindle. Time point of 0ms was arbitrarily defined to emphasize the example of the spindle-event that follows a random slow-wave. b. Representative average EEG slow wave (μV, top graphs) and Sigma amplitude (% , 9-13Hz, bottom graphs) of one young (left, black) and one old mouse (right, gray). c. Average Sigma amplitude (% , mean values $\pm$ SEM) in the young (black, n=9) and aged mice (gray, n=9). The asterisk indicates significant difference between young and old mice for the period of the trough (unpaired t-test, p=0.0286).

Slow-wave Incidence and Multipeak Waves

To investigate the origin of the differences in absolute SWA between young and old animals, we analyzed the incidence of individual slow waves, in five 2h values, during the BL sleep period and during sleep after SD. To this end, all slow waves were sub-divided into five amplitude quintiles ranging from 0-100%, as described previously [18, 19] within three time points during BL (0-2, 6-8, 10-12h) and two time points during sleep after SD (6-8, 10-12h) (Fig. 4a). No differences in the incidence of slow waves were found between the age groups. In both age groups, slow-wave incidence significantly decreased across the BL sleep period and significantly increased after SD, and this was observed for slow waves within each 20% quintile (2-way ANOVA, interaction factors 'time of day*amplitude levels'; p<0.0001, 'age*time of day'; p=0.275, 'age*amplitude levels'; p=0.428). Similar significant decreases were found between the first and last 2h values of the 6-h interval after SD.

In both groups, the number of multipeak waves (number of peaks within a single wave, five 2h values expressed as percentage of the total number of waves during each time point at the respective amplitude quintile) was lowest among the highest-amplitude slow waves, but invariably an increase in the course of the main sleep period was observed (Fig. 4b). An age-dependent difference was apparent, manifested in significantly lower proportion of multipeak waves in older animals, as compared to young controls (Fig. 4b,

2-way ANOVA, interaction factors 'age*time of day'; p=0.001, 'age*amplitude levels'; p=0.003). On average, in the aged mice 1.14 peaks per slow wave occurred, in the main sleep period, whereas, young controls had 1.22 peaks per slow wave (unpaired t-test (t, df)=(-5.491, 55.241); p<0.0001).

Previously, a negative correlation was found between the number of multipeak waves and SWA in young rats and humans [18, 19]. Since both variables showed significant changes in the course of aging, we conducted a regression analysis, to determine whether the relationship between multipeak waves and absolute SWA was influenced by aging. In both groups, 2h values obtained during the BL light period (six time points at 0-2, 2-4, 4-6, 6-8, 8-10, 10-12h) of the highest-amplitude multipeak waves (expressed as percentage of the total number of waves during each time point, at the 80-100 amplitude quintile) were significantly negatively correlated with the six corresponding 2h values of absolute SWA, obtained during the BL light period. (R^2 in young: 0.885; old: 0.952, Fig. 5). However, the age groups formed two distinct clusters where the slope (young: -0.3921 $\pm$ 0.0512; old: -0.2231 $\pm$ 0.0179; p=0.007), and intercept (young: 60.807 $\pm$ 3.005; old: 54.67 $\pm$ 2.84; p= 0.034) of the regressions differed significantly.

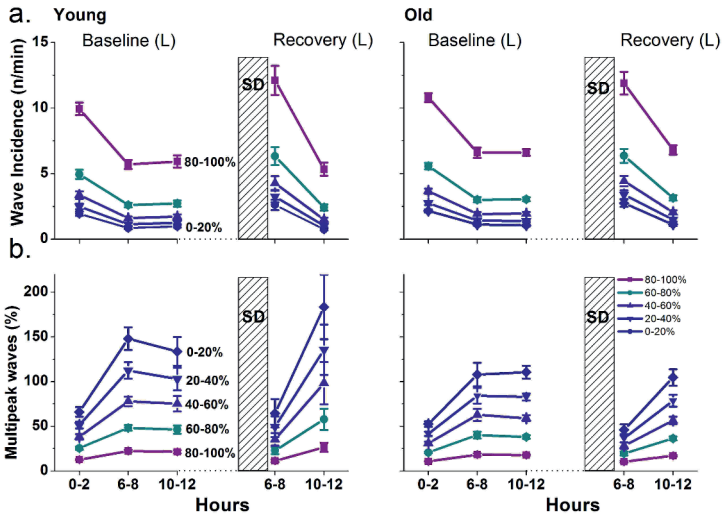

Figure 5.4: Slow-wave incidence (n/min) and number of slow waves with more than one peak (multipeak waves) during five 2-h time points across the light period of baseline and recovery for young (left, n=9) and old mice (right, n=9). Slow waves were equally subdivided into five amplitude quintiles ranging from 0-100%. a. The wave incidence (mean values ± SEM) changes significantly in the following time points in all amplitude quintiles in both groups: 0-2h vs. 10-12h in baseline, 6-8h vs. 10-12h in recovery, 6-8h in baseline vs. 6-8h in recovery (paired t-tests, $p<0.05$ after significant 2-way ANOVA, interaction factors 'time of day*amplitude levels'). b. Multipeak waves are expressed as a percentage of the total number of slow waves (mean values ± SEM). They change significantly in the following time points in all amplitude quintiles in both groups: 0-2h vs. 10-12h in baseline, 6-8h vs. 10-12h in recovery, 6-8h in baseline vs. 6-8h in recovery. Significant differences were found between the groups, with old mice having overall less multipeak waves (paired and unpaired t-tests, $p<0.05$ after significant 2-way ANOVA with interaction factors 'age*time of day' and 'age*amplitude levels').

Slow-wave slopes

Age-dependent differences in multipeak slow waves suggest that network synchronization may be altered in older animals. Therefore, next, we investigated the dynamics of changes in the slopes of the slow waves, so that conclusions can be drawn in the comparison with older humans [5, 6] as well as young rats [18]. The slope of the first and the second segment of the slow wave was computed in five 2h values across the BL sleep period (0-2, 6-8, 10-12h) and after SD (6-8, 10-12h) for the five amplitude quintiles (Fig. 6). The slope of the first segment (Slope 1, expressed as % of the 12h mean) did not show noticeable changes, in the course of the main sleep period, in the young mice, whereas, significantly steeper slopes were observed during early sleep in the old mice (Fig. 6a, 2-way ANOVA, interaction factors 'age*time of day'; $p<0.0001$). After SD, no significant change in the 1st slope was found in either group. The second segment of the slow-wave slope (Slope 2, expressed as % of the 12h mean) changed in the course of the BL sleep period in both age groups, however with a different time course (2-way ANOVA, inter-

action factors 'age*time of day'; p=0.001) (Fig. 6b). Steeper 2nd slopes were observed in the first 2h of the main sleep period in the highest-amplitude slow waves in the young (80-100% quintile) whereas in the older animals significant changes were found in all slow waves, irrespective of amplitude. After SD, steeper 2nd slopes were found only among the highest-amplitude slow waves in the young mice, whereas in the older mice slow-wave slopes were initially higher and subsequently declined, irrespective of slow-wave amplitude. No significant changes in slopes were obtained for the first 2h in BL vs. the first 2h of the recovery condition for the young mice, but steeper slopes were apparent for the highest-amplitude slow waves in the older mice. For the 6-8th h in BL vs. corresponding time after SD, significant steeper slopes were obtained after SD, in all amplitude quintiles, in both age groups. Overall, these findings reveal an effect of age on slow-wave slopes. Within the older group, we found a consistent generalized decrease in slopes in the course of the main sleep period among both low- and high-amplitude slow waves. In contrast, in the young mice, only the second slope of the high-amplitude slow waves (80-100% quintile) decreased significantly in the course of the day, whereas it was not the case for the slope of the lower-amplitude slow waves or the first slope.

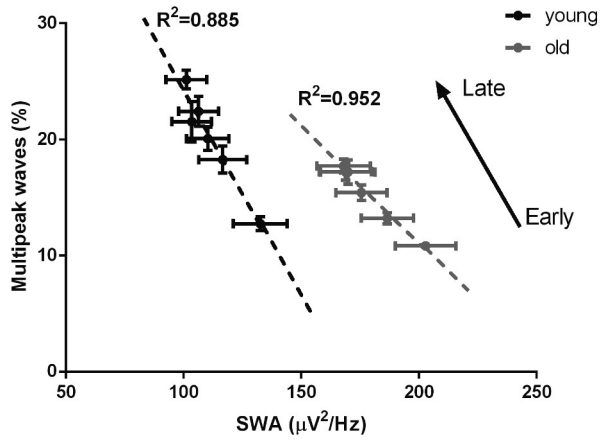

Figure 5.5: Relationship between absolute non-rapid-eye movement (NREM) sleep slow-wave activity (SWA, 0.5-4Hz) and multipeak waves (expressed as a percentage of the total number of slow waves) computed for the six 2-h intervals across the light period (time points 0-2, 2-4, 4-6, 6-8, 8-10, 10-12h) of baseline for young (black, n=9) and old mice (gray, n=9) for the highest-amplitude slow waves (80-100%) (mean values ± SEM). Dashed lines depict linear regression (R^2 are significantly different between young and old mice, unpaired t-test p=0.013).

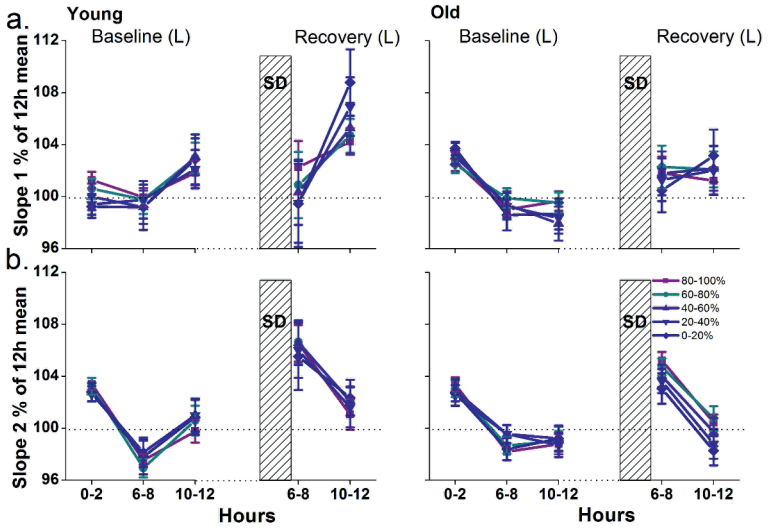

Figure 5.6: Slope of the first and second segment of the slow waves during five 2-h time points across the light period of baseline and recovery for young (left, n=9) and old mice (right, n=9). Slow waves were equally subdivided into five amplitude quintiles ranging from 0-100% and slopes (mean values ± SEM) are expressed as % of the 12h mean. a. The slope of the first segment (Slope 1) changes significantly in the 0-2h vs. 10-12h in baseline, only in the old mice in all amplitude quintiles (paired t-tests, $p<0.05$ after significant 2-way ANOVA with interaction factors 'age*time of day'). b. The slope of the second segment (Slope 2) changes significantly in the following conditions: 0-2h vs. 10-12h in baseline in young mice in the 80-100% quintile, 0-2h vs. 10-12h in baseline in old mice in all amplitude quintiles, 6-8h vs. 10-12h in recovery in the young mice in the 80-100% quintile, 6-8h vs. 10-12h in recovery in the old mice in all amplitude quintiles, 0-2h in baseline vs. 6-8h in recovery in the old in the 80-100% quintile and 6-8h in baseline vs. 6-8h in recovery in all amplitude quintiles in the young and old mice (paired t-tests, $p<0.05$ after significant 2-way ANOVA with interaction factors 'age*time of day').

Discussion

We conducted EEG and EMG recordings and performed a detailed analysis of individual EEG slow waves in healthy young and older mice, and show that older mice display significant changes in a broad range of sleep and EEG parameters, compared to young mice. Many of the changes suggest persistently elevated sleep pressure in the older mice. Similar to the effects after SD [33, 36, 45, 46] older mice slept more and had longer NREM sleep episodes during the dark period. Furthermore, older animals exhibited increased EEG power density during NREM sleep in slow frequencies (2-7 Hz) and, compared to young controls, had an overall higher level of NREM sleep SWA, which is also typical for increased sleep pressure [47, 48, 49]. Consistently, analysis of slow-wave characteristics revealed that older mice had fewer multipeak waves, steeper absolute slow-wave slopes and more pronounced changes in the relative slow-wave slopes, similar to the effects of sleep deprivation [18]. Interestingly, the modulation of sigma amplitude in association with the occurrence of individual slow waves was also stronger in older animals, sug-

gesting a tighter synchronization among cortical neuronal populations during both the network activity and silence. This is in line with increased sleep pressure, as it was previously shown, that network synchronization increases during sleep, under elevated sleep pressure [40]. Finally, the decay rate of SWA after SD was reduced in older mice, which is indicative of a slower recovery process. Altogether, the data suggest that brain network properties may be altered in older mice. We hypothesize that these changes result in a less restorative sleep, which prevents efficient dissipation of sleep pressure, and leads to a chronically sleep deprived state.

Sleep architecture is affected by aging

Older mice had increased NREM sleep in the dark period, and reduced REM sleep at the end of the light phase. These findings are in accordance with previous findings in older mice [7, 8, 9, 10, 11, 12]. For example, previous work in C57BL/6J mice, where 23 months old mice were compared to 3 months old [7] or 21 months old mice to 5 months old [9] or 22-24 months old to 2-4 months old [10] revealed increased NREM sleep in the Dark period and decreased REM sleep in the light. In another study [8], an increase in NREM and REM sleep from the age of 3 months to 1 year in DBA/2J, C57BL/6J mouse strains, and in NREM sleep in AKR/J mice, was reported, whereas at the age of 24months old a decrease in NREM was reported in the C57BL/6J and DBA/2J mice as compared to the 1 year old, and a decrease in REM sleep in the C57BL/6J mice as compared to the 1 year old and 6 months old mice and in the DBA/2J mice as compared to the 1 year old mice. Some studies, therefore, found a decrease in sleep in 2 year old mice compared to 1 year old mice, but most found a steady increase in sleep in the course of aging. None of the mouse strains at higher age (>18 months old) showed a decrease in sleep below the level of 3 months old. In general, we can conclude that our findings are in accordance with most studies regarding the C57BL/6J strain, but similar changes in sleep architecture were also found in other mouse strains.
Furthermore, we found in the older animals more long NREM sleep episodes and more short wake and REM sleep episodes during the dark phase compared to the young animals, similar to a previous study [10].
Sleep deprivation did not influence the differences in waking and NREM sleep between young and older mice, however, older mice showed a clear increase in REM sleep compared to BL in the dark period which was not observed in the young group.
The physiological mechanisms underlying age-induced changes in sleep architecture have yet to be determined. It was proposed that alterations in neurotransmitter and receptor balance, such as increased adenosine, a ‘sleep facilitator’ and decreased hypocretin (orexin), a ‘wakefulness regulator’, as well as alterations in the glutamatergic and GABAergic systems, can lead to sleep-wake dysregulation [50, 51, 52, 53]. Moreover, changes in the output strength of the circadian clock may underlie the changes seen in the course of aging [54]. In that context, the increase in the amount of sleep during the dark phase may be caused by lower circadian amplitude, causing a weaker waking signal coming from the SCN in the active period of the mice.

Aging is associated with changes in EEG power density

In contrast to humans [1, 2, 3, 5, 6, 37, 38], the EEG in older mice was characterized by higher absolute power density in the EEG slow wave range, as compared to young mice. In the waking spectrum, less power density was found in the 5-12 Hz frequency range and more power density in the lower frequencies around 3 Hz in the older mice. These findings were specific for the respective vigilance state and no differences were observed in the REM sleep spectrum. They are, therefore, not caused by a general unspecific change in the EEG activity of older mice, suggesting that NREM sleep EEG regulatory mechanisms are likely to be more affected compared to REM sleep in aging.
The difference in 5-12 Hz activity during waking can be explained by differences in the amount of active waking. In rodents, theta activity in the EEG is increased during active wakefulness, for example during exploration, compared to quiet waking [55, 56]. The lower theta activity in the older mice is in accordance with previous findings, showing possibly less exploratory behavior and reduced arousal in older compared to young mice [10]. Furthermore, the increase in EEG spectral power around 3 Hz in waking is reminiscent of the effects of sleep deprivation, and may reflect intrusions of sleep-like network activity into the awake state [21]. This, also, agrees with the notion that sleep pressure in older mice is elevated.
Older animals had an overall elevated level of SWA in the NREM sleep EEG (Fig. 1a). NREM sleep SWA is the best characterized marker of sleep intensity and increases in response to sleep loss16. It decreases in the course of the main sleep period and gradually increases in the dark period, when the animals are more awake and active, a pattern visible in both groups. However, older mice showed higher absolute SWA values across the whole recording period, with significant differences compared to the young animals, particularly in the light period and at the end of the dark period. These observations contrast human studies, where absolute SWA values, as well as absolute amplitude, frequency and density of slow waves are found to be decreased in aging [1, 2, 3, 5, 6, 37, 38].Moreover, the decay rate in SWA in the first three hours after SD was slower in the older mice compared to the young controls. The latter suggests that sleep in older mice is less efficient in dissipating sleep pressure. Previous studies in mice suggested that absolute levels of SWA correlate with homeostatic sleep pressure [47, 48, 49]. Therefore, the higher level of absolute SWA in our data supports the notion of an increased sleep pressure phenotype in older mice.

Age-dependent changes in SWA arise from changes in slow-wave morphology

To better understand the network mechanisms, underlying the differences in SWA between young and older mice, we assessed several characteristics of individual EEG slow waves, as it has been performed in earlier studies in the rat [18] and in humans [5, 6, 19]. The dynamics of slopes of the slow waves in old mice showed similar properties to the study conducted in the rat [18]. In young mice only the 2nd slope of the high-amplitude slow waves (80-100% quintile) changed significantly in the course of the day, whereas in older mice changes were found in all slow wave amplitude ranges both in the 1st and 2nd slopes. The time course of the changes in these slopes showed similar dynamics as the time course of SWA. Increases in the 2nd slope were also found after sleep deprivation and this is therefore an indication of increased sleep pressure in the old mice [18].

The absence of changes in the 1st slope, in the course of the main sleep period in the young mice, suggests a lower network synchronization into the silent state of the neurons in young mice, compared to the older mice. In middle-aged (41-60 y.o.) [5] and older humans (50-70 y.o.) [6], an age-related decrease in absolute slow-wave slope was found, in contrast to our data in mice.
The cellular counterpart of EEG slow waves - the slow oscillation - consists of an up state, when neurons are depolarized, and a down state, when neurons are hyperpolarized and spiking is diminished. Overall, NREM sleep SWA is a reflection of near-synchronous transitions between up and down states among the large population of cortical neurons near the recording electrode [39, 40, 43]. Evidence suggests that the more synchronous the firing pattern of single neurons is, the higher the amplitude of corresponding EEG slow waves, and the steeper the slow-wave slopes. Therefore, slow-wave slopes can be considered as indicators of neuronal synchronization and, indirectly, the synaptic strength in the cortex [18, 20]. Moreover, changes in the slow wave parameters are mediated by changes in the amplitude of single-cell oscillations, in the dynamics of network synchronization, and in the rate of neuronal recruitment and decruitment [20, 28]. Therefore, the increased absolute SWA and slow-wave slopes, the decreased sigma activity during the down state, and the decrease in multipeak waves that we found, possibly indicate that more neurons are recruited in producing the overall slow wave in the older mice, suggesting increased neuronal coupling at the local level between cortical neurons in this group. Consistent with previous studies [18, 19], in our data the incidence of slow waves is positively correlated with absolute SWA whereas multipeak waves follow an opposite pattern. Notably, older animals had fewer multipeak waves across the day compared to young controls. Multipeak waves reflect the activity of asynchronous spatially distinct clusters of neurons due to poor spatial synchronization when the overall connection strength is reduced20. In both age groups, the number of multipeak waves increased in the course of prolonged sleep, similar to previous data obtained in rats and humans [18, 19]. However, when analyzing the relationship between SWA and multipeak waves, the age groups separated into two clusters. Multipeak waves are independent of amplitude and, hence, this shows that our findings are not merely based on an amplitude difference between the two age groups, but that the morphology of the slow waves is indeed altered between the age groups. This, together with the difference found in sigma activity during the down state of the slow wave, suggests a consistent change in cortical network connectivity, which possibly develops in the course of aging. In young mice, high global synchronization probably occurs together with possibly relatively lower local one, ending up in an increased number of up states and down states that effectively cancel each other out. At the cortical level, the relative synchronization observed during slow-wave sleep is likely the result of the high interconnectivity of the thalamocortical system [20]. Taking into account that age-related sleep impairments are unlikely to be due to neuronal loss, but due to changes in synaptic connectivity [57] and, thus, loss in neuronal connections, our data suggest neuronal connectivity changes in the cortical network, in the course of aging, possibly towards weaker global and stronger local synchronization. However, as we did not measure unit activity of neurons, more research is needed to investigate this further.
Recent research, applying in-vivo two-photon imaging that tracked the size and location of axonal boutons in aged mice, revealed an increase in synaptic structural dynamics in

specific cortical circuits in the aged cortex [58]. Increased rates of synapse formation, elimination and destabilization, and decreased synaptic tenacity in the aged cortex could potentially cause the slow wave alterations that we detect through EEG recordings reflecting the increased network connectivity in local circuits.
Furthermore, it has been shown in aged mice that mean postsynaptic density synapse length is greater and that there is selective maintenance of large prefrontal spines with reduced plasticity [59]. Both aforementioned studies suggest alterations in the aging cortex regarding the synapse and the structural connectivity and plasticity that may lead to the increased network connectivity in local circuits suggested by our results.

From mice to humans

Although at a first glance the changes in cortical network connectivity in the course of aging seem to differ between humans and mice, further research, from large-scale electric signals to single-cell recordings, is undoubtedly necessary. The EEG electrodes used in mice and humans differ significantly in size, and so does the size of the neuronal population recorded. It is possible that intracerebral recordings in humans, where a small network contributes to the field potential picked up by the electrode, may reveal similar differences to those found in mice. Volume conduction may have a considerably greater influence on the EEG waveforms in humans. The possibility remains that long-distance connectivity is reduced, both in humans and mice, but this occurs on a spatial scale, which cannot be compared directly. In an earlier study in humans [3] age groups: 20-30 y.o, 31-45 y.o., 45-60 y.o.) [60], an increase in EEG dimensional complexity was revealed with age, supporting the hypothesis that the complexity of brain dynamics increases with brain maturation. It was argued that this may be caused by an increase in the number of functionally related cell assemblies oscillating at their own frequencies. This, may be the equivalent to the increase in slope and amplitude found in NREM sleep slow-waves in the older mice.
Furthermore, dysfunctional circadian rhythms potentially related to sleep-wake disturbances have been reported as an explanation of the changes found in sleep as humans age. In our results, the most profound differences between the age groups were found in the dark phase of the day, the active period of nocturnal animals, which is in accordance with previous reports [7, 8, 9, 10, 11, 12]. With age, NREM sleep, and especially the longer episodes, increased during the night. This could be equivalent to the increased daytime sleepiness and decreased sleep at night described in elderly humans [61, 62, 63]. These changes in sleep architecture in humans and mice may be related to a decrease in the amplitude of the circadian clock [54]. Since SCN neuronal activity is similar in diurnal and nocturnal animals, this results in increased sleep in the active period in mice and decreased sleep in the rest period in humans.
Contrary to human data [1, 2, 3, 5, 6, 37, 38], aged mice demonstrated an increment in the absolute SWA, amplitude and slow-wave slopes. The aged mice in our study were between 18-24 months old, which is approximately equivalent to 50-70 years in humans. The human subjects in the studies that investigated the slow-wave characteristics, in parallel with the SWA, were middle aged (41-60 years of age) [3, 5] or older (50-70) [6]. It is, therefore, unlikely that the differences between humans and mice are caused by an inappropriate age match. Therefore, in the course of aging, the cortical network seems

to undergo changes which are reflected in the NREM sleep EEG. These EEG changes support the notion of a higher sleep pressure in the aged mice, contrasting what has been found in the elderly humans. This, does not render the mouse model, specifically the C57BL/6J strain, invalid for sleep and aging research, however, these specific differences have to be taken into account when comparing between species.

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Supplementary Material

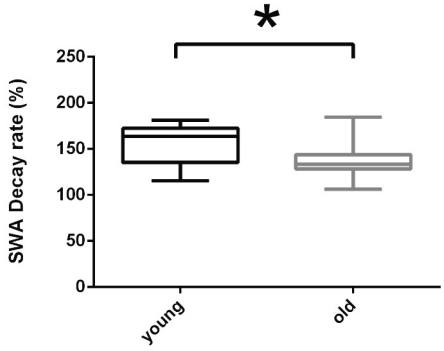

Figure 5.1: Decay rate of slow-wave activity (SWA) for the first three hours after sleep deprivation (SD) for young (black, n=9) and old mice (gray, n=24). The asterisk indicates significant difference between the two age groups (unpaired t-test, p=0.038).

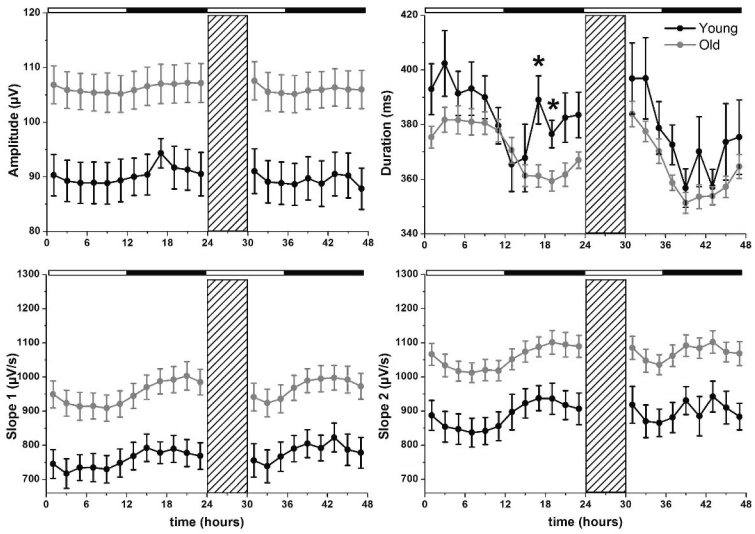

Figure 5.2: Time course of absolute slow wave amplitude, duration, first and second slope of the highest-amplitude (80-100%) slow waves for 24-h baseline, 6-h sleep deprivation (SD, hatched bar) and 18-h recovery for the two age groups, young (black circles, n=9) and old mice (gray circles, n=9). Curves connect 2-h values (mean ± SEM), black and white bars indicate the light-dark cycle and asterisks indicate significant differences between young and old animals (unpaired t-tests, $p<0.05$ after significant ANOVA). For the amplitude, duration and slopes higher absolute values were observed over the entire 48h recording period (one-way ANOVA, factor 'age'; $p<0.0001$).

Chapter 6

Exercise, sleep and brain age

How old is your brain? Slow-wave activity in non-rapid-eye-movement sleep as a marker of brain rejuvenation af-ter long-term exercise in mice

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Abstract

Physical activity is beneficial for health. It has been shown to improve brain functioning and cognition, reduce severity of mood disorders, as well as facilitate healthy sleep and healthy aging. Sleep has been studied in healthy aged mice and absolute slow-wave-activity levels (SWA, electroencephalogram power between 0.75-4.0 Hz) in non-rapid-eye-movement sleep (NREM) were elevated, suggesting changes in brain connectivity. To investigate whether physical activity can diminish this aging-induced effect, mice of three age groups were provided with a running wheel (RW) for 1-3 months (6-months-old, n=9; 18-months-old, n=9; 24-months-old, n=8) and were compared with control sedentary mice (n=11, n=8 and n=9 respectively). Two weeks before the sleep-wake recordings the running wheels were removed. The electroencephalogram (EEG) and electromyogram were continuously recorded during undisturbed 24h baseline (BL) and a sleep-deprivation was conducted during the first 6h of the second day. Increased waking and decreased NREM sleep was found in the young RW mice, compared to young controls. These effects were not evident in the 18 and 24 months old mice. Unlike sleep architecture, we found that SWA was altered throughout the whole age spectrum. Notably, SWA was increased with aging and attenuated with exercise, exhibiting the lowest levels in the young RW mice. To utilize the cross-age revealing features of SWA, we applied machine learning techniques and found that characteristic information regarding age and exercise was enclosed in SWA. In addition, with cluster analysis, we could classify and accurately distinguish the different groups based solely on their SWA. Therefore, our study comprises a three-fold contribution: a) effects of exercise on sleep are sustained following two weeks after removal of the wheel, b) we show that EEG SWA can be used as a physiological marker of brain age in the mouse, c) long-term voluntary regular age-matched exercise leads to a younger phenotype.
Keywords: EEG, SWA, spectral analysis, running wheel, exercise, aging

Introduction

Sleep is a vital physiological routine that has a critical function in promoting the health of all living organisms. It is regulated by two processes, the circadian and homeostatic process [1, 2]. The circadian process is controlled by the circadian pacemaker, which is located in the suprachiasmatic nucleus and fluctuates with a cycle of approximately 24 hours, promoting wakefulness during the daily active phase and sleep during the rest phase. The homeostatic process refers to the sleep drive that builds-up during wakefulness and is dissipated during sleep. In mammals, sleep homeostasis is thought to be reflected in electroencephalographic slow-wave activity during non-rapid-eye-movement (NREM) sleep [2].

In healthy aged humans, several sleep parameters deteriorate and the overall sleep quality is impaired [3, 4, 5]. To further elucidate this disruption, animal models have been used to investigate sleep in the course of aging [6, 7, 8, 9, 10, 11, 12]. It was recently demonstrated that slow-wave activity in the NREM sleep electroencephalogram (EEG SWA) was substantially increased in aged, as compared to young mice. In addition to the increased SWA levels, the overall morphology of the slow-waves was shown to be changed, suggesting cortical brain alterations [11].

Sleep disorders, commonly encountered in aging, can be rectified with the use of pharmacological remedies [13, 14]. Although pharmacology often comprises the first-order treatment, lifestyle interventions such as exercise, have been proposed as a potential to ameliorate sleep disturbances [15]. Regular physical activity is, generally, considered to be beneficial for health [16]. Aerobic exercise, such as running, has been demonstrated to have a role in brain function and cognition, in preventing and treating or reducing severity of stress-related mood disorders, as well as in promoting healthy sleep cycles and healthy aging [17, 18, 19]. The effect of regular physical activity in parameters such as cognitive functioning and performance in elderly people, as well as aged animals, has been thoroughly investigated and found positive [20, 21]. However, the long-term impact of regular exercise on sleep and EEG SWA levels remains largely uncharted in the course of aging.

Concretely, acute and chronic effects of exercise on sleep have been investigated in young humans [22]. However, detailed studies in elderly humans are scarce and mainly demonstrate a more moderate effect of regular exercise on sleep quality compared to young subjects [23, 22, 24]. In rodent-based animal models, analogous findings are also limited. Access to exercise equipment, such as running wheels, as an equivalent to treading mills in humans, has shown beneficial effects on sleep in young mice [25], and young and aged F344 rats [26].

In the current study, we investigated the effect of regular physical activity on sleep architecture and EEG SWA of male C57BL/6J mice of three different age groups (6, 18 and 24 months old). For this, a running wheel was introduced in the cage of the mice that could be used voluntarily on a daily basis for 1-3 months. The wheels were permanently removed two weeks prior to the sleep recordings, therefore, a sustained effect of physical activity on sleep was profoundly studied. Based on our previous results, showing a lower daily amplitude in sleep-wake distribution and an increase in SWA in NREM sleep in the course of aging [11], we hypothesized a beneficial effect following long-term exercise consisting of a sleep pattern amelioration and lower SWA in NREM sleep in these mice.

Materials and Methods

Animals

Male C57BL/6JOlaHsd mice of three age groups (6, 18 and 24 months old, n=54) (Harlan, Horst, the Netherlands) were used for this study. They were partitioned into six distinct groups. Three control groups in which mice were sedentary throughout their life (n=11, 8, and 9 with increasing age respectively) and three running-wheel (RW) groups where a running wheel was introduced in the cage (n=9, 9 and 8 with increasing age respectively), for voluntary use in a daily basis in order for the mice to be exposed to a type of aerobic exercise as preferred in human studies. Aerobic exercise, also characterized as endurance activity or cardiovascular exercise, consists of a sustained period of rhythmic movement of large muscles, in which pumping of oxygenated blood by the heart is required so as oxygen is delivered to the muscles to generate energy. A beneficial effect of specifically aerobic fitness training on brain function and cognition has been explored [17]. In particular, for the 6 months old the access lasted for approximately a period of 1 month, and for the two aged groups for at least 3 months, prior to the sleep recordings. Therefore, young mice had access to the running wheel from the age of 4-5 months, while 18 and 24 months old mice had access to the running wheel from the age of 15 and 21 months respectively, prior to the sleep recordings (Fig. 1a). Throughout the text we often refer to the running wheel activity as "exercise". The mice were individually housed under controlled conditions (12:12 h light:dark cycle; lights on at 10:00) with food and water ad libitum in a temperature controlled room ($\sim$ 21-22 °C). All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Centre (the Netherlands) and were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Behavioral Activity recordings

The voluntary RW animals were individually housed in cages with a running wheel, to record their behavioral activity pattern. The number of wheel revolutions was digitized in 1 min bins with a computerized recording system (Clocklab; Actimetrics). Behavioral activity was plotted in actograms, and the circadian period, the amount of wheel running per 24-h, as well as the strength of the circadian rhythm, defined as the difference between the 95% confidence limit and the peak in the periodogram, was calculated with F-periodogram analysis, as described previously [27, 28], using the Clocklab analysis software (Actimetrics, Wilmette, IL). Control animals, without a wheel, were housed individually in similar cages, for the same duration.

Surgeries

On the day of the surgery, the running wheel was permanently removed from the cages of the animals. Before the surgeries, 6, 18 and 24 month old RW mice weighed on average 24.4, 36.9 and 38.5g respectively and 6, 18 and 24 month old control mice 30.7, 33.5, 32.7 g respectively. Under deep anesthesia (Ketamine 100 mg/kg; Xylazine 10 mg/kg; Atropine 1 mg/kg) EEG recording screws and electromyogram (EMG) electrodes (Plastics One) were implanted as described previously [29, 11]. One EEG electrode

was placed over the right hemisphere (2 mm lateral to the midline of the skull, 2 mm posterior to bregma) above the somatosensory cortex and the other was placed above the cerebellum (midline, 2 mm caudal to the lambda) as a reference. EMG electrodes were placed on the neck muscle. The wire branches of all electrodes were set in a plastic pedestal (Plastics One, Roanoke, VA) fixed to the skull with dental cement. The mice were allowed to recover for 7-10 days.

EEG recordings

The EEG and EMG were recorded with a portable recording system (PS 1 system, Institute of Pharmacology and Toxicology, Zurich, Switzerland) as previously described [11, 29, 30]. Before each recording, a calibration signal was recorded on the EEG and EMG channels. Both signals were amplified, conditioned by analogue filters and sampled at 512 Hz. The signals were filtered through a digital finite impulse response filter and stored with a resolution of 128 Hz. EEG spectra were computed for 4-s epochs. To record the EEG and EMG, animals were placed into experimental chambers and connected through a flexible cable and a counterbalanced swivel system to the recording setup. Conditions in the experimental chamber were similar to the home cage, without running wheel availability. Before starting the experiment, the animals were allowed to adjust to the experimental conditions for a week. Subsequently, a baseline (BL) day was recorded, starting at lights on. At the start of the second day, six hours of sleep-deprivation (SD) were conducted by gentle handling [11, 29, 31]. During that period, every time the animals appeared drowsy, or the EEG exhibited slow waves, the animals were mildly disturbed by noise, movement of bedding, or introducing new nesting material or food into the cage. EEG and EMG were recorded continuously during SD and, subsequently, for 18 hours to investigate sleep characteristics after SD. The time between the day of the surgery and the BL sleep recordings was approximately 2 weeks. All animals contributed once to the sleep recordings and no animals were double used.

Data analysis and statistics

Three vigilance states (Waking, NREM sleep and REM sleep) were scored offline in 4s epochs by visual inspection of the EEG and EMG signals as well as EEG power in the slow-wave range, as described previously [11, 29, 31]. For each epoch, the EEG power in the delta (0.75-4.0 Hz) and theta band (6.25-9.0 Hz) and the integrated EMG value were graphically displayed on a PC monitor to enable scoring of the different vigilance states (waking, NREM sleep and REM sleep). The vigilance states were expressed as a percentage of artifact-free recording time. Epochs with artifacts were excluded from the subsequent data analysis of the power spectra, but vigilance states could always be determined. Spectral analysis was performed using a fast Fourier transform (FFT; 0.5-25 Hz). Analysis of variance (ANOVA) with factors ‘exercise’ or ‘strength’ was performed to detect differences in daily running wheel activity amount and strength of the 24-h rhythm respectively across the age groups. Three-way ANOVA with factors ‘treatment’, ‘time of day’, ‘day’ for 2-h vigilance states and EEG SWA and two-way ANOVA with factors ‘treatment’ and ‘Light-dark’ for 12-h vigilance states and ‘treatment’ and ‘frequency bin’ for EEG spectra was applied to test the differences between the groups and experimental conditions. One-way ANOVA factor ‘group’ was applied to detect differences across six

groups in EEG SWA in 12h BL light period. Additionally, to test whether SD induced alterations in EEG SWA, for each group separately a two-way ANOVA was performed with factors 'time of day' and 'day'. When appropriate, paired and unpaired post hoc Bonferroni-corrected student's t-tests were applied to determine the effects of exercise (treatment) or SD.

Profiling and classifying slow-wave activity

We performed additional statistical analysis on the SWA data. First, we used a supervised learning approach to examine if the SWA data contained sufficient information to correctly classify the different groups based on their age and the availability of the running wheel. Next, an unsupervised learning approach was taken to identify, in an unbiased fashion, clusters from the data. The distances between the central points of the identified clusters (centroids) show the similarity of the SWA between clusters; a larger distance signifies a larger difference in SWA. For our analysis, we used SWA values, averaged over 1-h intervals, obtained during the 12h BL light period, resulting in a 12-dimensional signal for every mouse. Matlab was used for the following analysis (The MathWorks, Inc., Natick, MA).

Supervised learning approach

We examined whether the data convey information about the age and the exercise of the mice using the generic perceived information metric [32, 33]. To acquire this metric we used profiling techniques (supervised statistical machine learning) that originate from signal processing [36] and have been successfully used for classification purposes in the context of EEG brain activity [37]. Subsequently, we tested whether this available information could be concretely used in order to find the previously unknown age and exercise level of a mouse. The effectiveness of this classification stage was quantified using the stage's success rate metric.

Throughout this section, capital letters denote random variables, small case letters denote their instances and calligraphic letters denote sets. Bold font denotes vectors and matrices. The slow wave activity is denoted as random variable $\mathbf{S}$ over alphabet $\mathcal{S} = \mathbb{R}^{12}$ and a particular SWA signal as $\mathbf{s}$. Similarly, we describe age with random variable A over $\mathcal{A} = \{6, 18, 24\}$ months and exercise with random variable E over $\mathcal{E} = \{\text{yes}, \text{no}\}$. We, also, define the age/exercise class with random variable $C = (A, E)$ over all possible age/exercise pairs $\mathcal{C}$, e.g. the 6-month-old mice that exercise are described by class $c = (6, \text{yes})$.

We grouped together the SWA signals of all mice that belong to a certain age/exercise class c, referring to it as the dataset $\mathcal{D}$ with respect to class c, or $\mathcal{D}^c$ for short. The grouping process was repeated for all $c \in \mathcal{C}$. Our supervised approach required separate datasets for profiling and testing, thus we partitioned $\mathcal{D}^c$ to $\mathcal{D}_p^c$ and $\mathcal{D}_t^c$, denoting the profile subset and the test subset respectively.

The profiling step of the classification technique uses the profiling dataset $\mathcal{D}_p^c$ to estimate a probabilistic model that describes the behavior of class c. Specifically, we assumed that for a given age/exercise class c, SWA follows a multivariate normal distribution, i.e. $Pr[\mathbf{S} = \mathbf{s}|C = c] \sim \mathcal{N}(\boldsymbol{\mu}_c, \boldsymbol{\Sigma}_c)$. We used $\mathcal{D}_p^c$ to estimate the mean $\boldsymbol{\mu}_c$ and the covariance

matrix $\mathbf{\Sigma}_c$ of the distribution, resulting in the probabilistic model $\hat{Pr}_{model}[\mathbf{S} = \mathbf{s}|C = c]$, also known as the template of c. Note that prior to parameter estimation, we performed dimensionality reduction on $\mathcal{D}_p^c$ via principal component analysis (PCA) on the SWA signals in order to extract most of the available information, while preventing the model training process from becoming too data intensive.
The test step of the classification technique uses the SWA signals of test dataset $\mathcal{D}_t^c$. Computing the perceived information (PI) metric requires approximating the true and unknown distribution $Pr_{true}[\mathbf{S} = \mathbf{s}|C = c]$, which can be sampled from $\mathcal{D}_t^c$. Computing the success rate metric requires comparing all signals in $\mathcal{D}_t^c$ against all class templates and finding the best-matching one.

Perceived Information

The Perceived Information (PI) can quantify the amount of information that the SWA $\mathbf{S}$ contains about the class C, taking into account the divergence between the real and the estimated models. Rephrasing, $PI(\mathbf{S}; C)$ shows how much the class entropy $H(C)$ is reduced, when information about SWA becomes available under imperfect modeling. A positive value of $PI(\mathbf{S}; C)$ indicates a sound SWA model that should lead to the successful classification of a mouse with unknown age/exercise. Note that the computation of PI requires the estimated model $\hat{Pr}_{model}[C = c|\mathbf{S} = \mathbf{s}]$, as well as the true distribution $Pr_{true}[\mathbf{S} = \mathbf{s}|C = c]$ of SWA, which is unknown and can only be sampled directly from the test dataset. Thus, the PI takes into account the model assumption and estimation errors that are introduced by the multivariate normal assumption and the respective parameter estimation [33]. If the true and the estimated distribution are very close, then the PI metric is equivalent to classic mutual information. The PI metric was computed as follows:

$$PI(\mathbf{S}; C) = H(C) - H(C|\mathbf{S}) = H(C) + \sum_{c \in \mathcal{C}} Pr[c] \sum_{\mathbf{s} \in \mathcal{D}_t^c} Pr_{true}[\mathbf{s}|c] * log_2(\hat{Pr}_{model}[c|\mathbf{s}]) d\mathbf{s}$$

$\hat{Pr}_{model}[c|\mathbf{s}] = \dfrac{\hat{Pr}_{model}[\mathbf{s}|c]}{\sum_{c^* \in \mathcal{C}} \hat{Pr}_{model}[\mathbf{s}|c^*]}$, using the Bayes' rule and $\hat{Pr}_{model}[\mathbf{s}|c]$ is estimated from $\mathcal{D}_p^c$

$Pr_{true}[\mathbf{s}|c] = \dfrac{1}{n_t^c}$, where n_t^c is the number of signals in test dataset $\mathcal{D}_t^c$, i.e. it is sampled from $\mathcal{D}_t^c$

To verify that our SWA classification procedure is accurate, we made use the cross-validation technique [34], which estimates how well our profiled model performs in practice. In particular, we we applied a 'leave-one-out' strategy, which is justified by the limited number of samples available in our experiments. The cross-validation was performed as follows:

1. The dataset $\mathcal{D}$ is split to $|\mathcal{D}|$ non-overlapping sets $\mathcal{D}(i), i = 1, \ldots, |\mathcal{D}|$, each containing a single SWA signal that originates from a single mouse.

2. For certain index i we defined a test set $\mathcal{D}_t = \mathcal{D}(i)$ and profile set $\mathcal{D}_p = \bigcup_{j \neq i} \mathcal{D}(j)$. Thus, we selected a single SWA signal denoted by index i for testing and used the rest for profiling. The set $\mathcal{D}_p$ was further partitioned to the corresponding class profile sets $\mathcal{D}_p^c,\ c = 1, \ldots, |\mathcal{C}|$.

3. For the given partitioning of (step 2), we computed $PI(\mathbf{S}; C)$, where $\hat{Pr}_{model}[\mathbf{s}|c]$ was estimated from $\mathcal{D}_p^c$ and $Pr_{true}[\mathbf{s}|c]$ was sampled from $\mathcal{D}_t$.

4. Steps 2 and 3 were repeated for all possible partitions of $\mathcal{D}$ to profile and test datasets which in the case of 'leave-one- out' strategy is $|\mathcal{D}|$ partitions. The PI estimates for every partition of step 2 were averaged and their variance showed the accuracy of the results.

We completed the perceived information approach by computing a confidence interval for the PI estimates. Specifically, we used a 10% bootstrap confidence interval [34] where we resampled 100 out of all the computed PI values (step 4), sorted them and set the confidence interval endpoints at the 5% and 95% percentiles.

Success Rate

The success rate metric quantified how successful our profiled model (template) was in recognizing the age/exercise class of mice that were not part of the profiling group ($\mathcal{D}_p^c$, thus expressing the classification's accuracy as a percentage). We employed the success rate metric to confirm that a positive PI also leads to a correct classification. For every class $c \in C$, we partitioned again $\mathcal{D}^c$ to profile and test datasets $\mathcal{D}_p^c, \mathcal{D}_t^c$, this time using a 50-50 ratio, instead of the 'leave-one-out' strategy. We split the dataset ($\mathcal{D}^c$) in two roughly equal subsets, in order to show that even fairly small profiling sets (just 50% of $\mathcal{D}^c$) can provide accurate classification and high success rate. The profile datasets $\mathcal{D}_p^c,\ c \in \mathcal{C}$ were used to estimate $|\mathcal{C}|$ SWA templates $\hat{Pr}_{model}[\mathbf{S}|c]$. Consequently, SWA signals from all test datasets $\mathcal{D}_t^c,\ c \in \mathcal{C}$ were compared with the templates using the maximum likelihood principle to find the class c that is the best match. Thus, for all classes $c \in \mathcal{C}$ and corresponding signals $\mathbf{s} \in \mathcal{D}_t^c$, we computed $argmax_c \hat{Pr}_{model}[\mathbf{s}|c]$. Matching a signal from set $\mathcal{D}_t^c$ with any template other than the template of c, constituted misclassification, that was reflected on the success rate.

Unsupervised Learning Approach

Note that the class labels assigned to mice, i.e., age, exercise, are closely coupled with our experimental design and hypotheses; we implicitly assumed 6 distinct mouse groups and tried to distinguish between them. However, this artificial separation into groups may not be in accordance with the information present in the dataset, e.g., certain groups may present strong similarities. To investigate this, we expanded our analysis with unsupervised learning approaches that take out all the assumptions of the researcher and provide an unbiased look at the data. In other words, this approach can find the most reasonable clustering in the data without using prior labeling and gauge the similarities between different clusters.
In order to estimate the optimal number of clusters in the SWA data, we used the 'Gap' statistic approach [35]. In this method, each cluster has a central point, named the centroid, and k-means clustering was used to compute the distances between the centroids of the clusters that were formed. The distance between the centroids is an indication of

the similarity of the SWA characteristic features between the clusters.
To be precise, a number (k) of observations was randomly chosen as being prototypes for the clusters. Then, using the Euclidean distance function, the SWA patterns for all experiments (after PCA-based dimensionality reduction, from twelve to three dimensions) were appointed to the cluster where the distance function was minimal. Subsequently, the centroid was calculated for each cluster. Then individual samples were reappointed to another cluster and the centroids were re-calculated. The procedure was repeated 1000 times to reach convergent behavior.
The Gap statistic approach uses first the result of k-means algorithm to compute the within-cluster sum of square around the cluster means. Second, this is compared to a reference distribution that is sampled from the dataset, which in turn leads to a score Gap[k]. Gap[k] score computation is used in order to evaluate the optimal number of clusters The k-means algorithm and the Gap[k] score computation are repeated for k = 1 until 6 clusters and the number of clusters k that optimizes the score is chosen.

Results

Running Wheel: behavioral data

Examples of 28 days running wheel activity from a 6-month, an 18-month and a 24-month old mouse are shown in Fig. 1b. Young mice showed significantly increased average daily running wheel activity compared to aged mice (6 months old: 27746 $\pm$ 1974 counts/24h, 18 months old: 5190 $\pm$ 2521 counts/24h, 24-months old: 6458 $\pm$ 1884 counts/24h) [One-way ANOVA, factor 'exercise', $F(2,23)=34.91$; $p<0.0001$, post hoc Bonferroni tests: $p<0.0001$]. Additionally, aging reduced significantly the strength of the 24-h rhythm (6 months old: 0.47 $\pm$ 0.02, 18 months old: 0.16 $\pm$ 0.05, 24-months old: 0.22 $\pm$ 0.05) [one-way ANOVA, factor 'strength', $F(2,23)=16$; $p<0.0001$, post hoc Bonferroni tests: $p<0.001$].

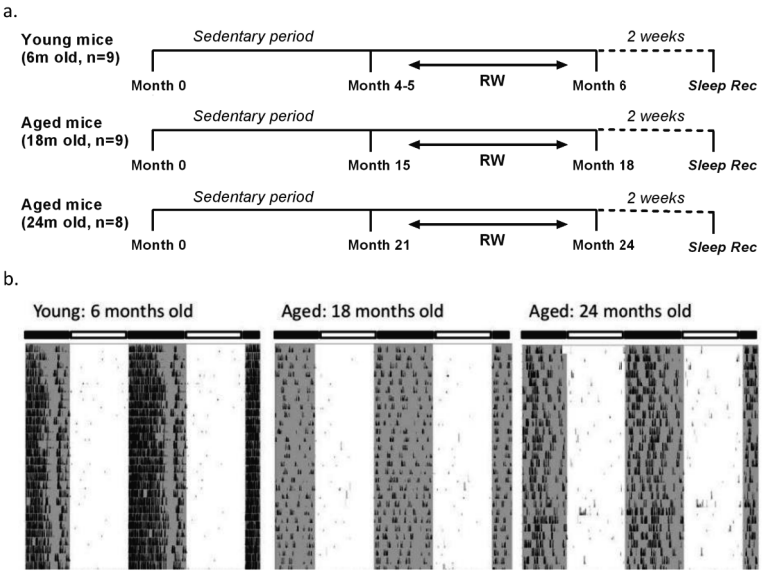

Figure 6.1: a. Schematic overview of the experimental design. Mice were provided with a running wheel in their cage for long-term daily voluntary use of approximately 1 month (young group) and 3 months (18 and 24 months old groups) prior to the sleep recordings. (RW: running wheel; Sleep rec: sleep recordings) b. Representative double-plotted actograms of 28 days running wheel activity from a 6-month, an 18-month and a 24-month old mouse. Young mice showed an increased average daily running wheel activity as compared to aged mice (6 months old: 27746 $\pm$ 1974 counts/24h, 18 months old: 5190 $\pm$ 2521 counts/24h, 24-months old: 6458 $\pm$ 1884 counts/24h) (post-hoc t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effect 'exercise').

Sleep architecture

During undisturbed BL, young mice that had access to the running wheel prior to the sleep recordings, showed increased waking and decreased NREM sleep in the first 6-h of the dark period, as compared to age-matched controls (Fig. 2) (post-hoc two-tailed unpaired t-tests between groups with Bonferroni multiple comparisons correction following significant interaction and main effects 'treatment', 'time of day' and 'day') (statistical analysis can be found in Table 1). The latter was also evident after SD. No effects were found in REM sleep. A more moderate effect of regular activity was evident in the older mice, that was reflected in a reduction in BL REM sleep at the end of the dark period in the 18 months old mice and during the light period in the 24 months old mice (Table 1). SD did not result in additional vigilance state alterations in the young group. This was also the case for the 24 months old mice (Table 1). In the 18 months old mice that had the running wheel, the ANOVA showed a stronger increase in NREM sleep and decrease in waking, compared to age-matched controls, however these effects did not survive post-hoc multiple comparisons correction (Table 1). Compared to BL, the period after SD was associated with increased NREM sleep and REM sleep and reduced

waking in all six groups (paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after aforementioned significant ANOVAs reported in table 1). Young 6-month old mice were more awake during the dark period and had less NREM sleep compared to older mice (Table 2). In addition, young exercising mice were even more awake and had less NREM sleep compared to young control mice (Table 1) [post-hoc unpaired t-tests between groups in specific light-dark periods: $p\leq0.05$]. The amount of REM sleep gradually decreased in the BL light period as a function of aging in the groups provided with a running wheel, where the 24 months old mice that had access to a wheel had the lowest amount of REM sleep in the BL light period among all groups (Table 1) [post-hoc unpaired t-tests between groups in specific light-dark periods: $p\leq0.05$].

EEG power density

The absolute EEG power density spectrum was computed for the three vigilance states in all groups (Fig. 3). The most pronounced differences in the waking and NREM sleep spectra were found in the young mice, where previous access to a running wheel decreased absolute EEG power density values in the slow frequencies (1-5 Hz) in waking and NREM sleep (post-hoc two-tailed unpaired t-tests between groups with Bonferroni multiple comparisons correction following significant interaction and main effects 'treatment' and 'frequency bin') (Table 1). Similar decreases in the slow wave range, albeit less robust, were found in the 18 months old mice in waking and NREM sleep and in 24 months old mice in NREM sleep (Table 1). Notably, groups previously provided with a wheel demonstrated decreases in absolute power density values in the slow EEG frequencies during REM sleep (0.5-5.5 Hz), albeit not always surviving the multiple comparisons correction. Theta activity (7-8 Hz) was increased in the young exercising animals, but decreased in the older age groups who had a wheel, compared to age matched controls (Table 1).

Table 6.1: Statistical analysis: two or three-way analysis of variance (ANOVA) was conducted to test the effect of exercise (treatment) and sleep deprivation (see text for more details). Asterisks following reported p values indicate significance (* p<0.05).

Table 1. Statistical Analysis			
Figure/Table	Vigilance state	Two- or three-way ANOVA Interaction factors	Main factors
Figure 2 (Young mice)	Waking	'treatment*day*time of day' F(11,432)=0.769 p=0.671 with 'treatment*time of day' p<0.0001*	'treatment' p<0.0001* 'day' p<0.0001* 'time of day' p<0.0001*
	NREM sleep	'treatment*day*time of day' F(11,432)=0.782 p=0.658 with 'treatment*time of day' p<0.0001*	'treatment' p<0.0001* 'day' p<0.0001* 'time of day' p<0.0001*
	REM sleep	'treatment*day*time of day' F(11,432)=0.527 p=0.885 with 'treatment*time of day' p=0.032*	'treatment' p=0.056 'day' p<0.0001* 'time of day' p<0.0001*
Figure 2 (18 months old mice)	Waking	'treatment*day*time of day' F(11,360)=0.898 p=0.542	'treatment' p=0.026* 'day' p<0.0001* 'time of day' p<0.0001*
	NREM sleep	'treatment*day*time of day' F(11,360)=0.821 p=0.619	'treatment' p=0.003* 'day' p<0.0001* 'time of day' p<0.0001*
	REM sleep	'treatment*day*time of day' F(11,360)=0.565 p=0.857	'treatment' p=0.032 * 'day' p<0.0001* 'time of day' p<0.0001*
Figure 2 (24 months old mice)	Waking	'treatment*day*time of day' F(11,354)=1.722 p=0.067	'treatment' p=0.825 'day' p<0.0001* 'time of day' p<0.0001*
	NREM sleep	'treatment*day*time of day' F(11,354)=2.041 p=0.024 *	'treatment' p=0.726 'day' p<0.0001* 'time of day' p<0.0001*
	REM sleep	'treatment*day*time of day' F(11,354)=0.741 p=0.699 with 'treatment*time of day' p=0.008* and 'treatment*day' p=0.005*	'treatment' p=0.02* 'day' p<0.0001* 'time of day' p<0.0001*
Table 2 (Young mice)	Waking	'treatment*Light-Dark' F(5,96)=4.773 p=0.001*	'treatment' p=0.008* 'Light-Dark' p<0.0001*
	NREM sleep	'treatment*Light-Dark' F(5,96)=4.1 p=0.002*	'treatment' p=0.002 * 'Light-Dark' p<0.0001*

Table 2 (24 months old mice)	REM sleep	'treatment*Light-Dark' F(5,96)=3.8 p=0.004*	'treatment' p=0.007* 'Light-Dark' p<0.0001*
Figure 3 (Young mice)	Waking	'treatment*frequency bin' F(29,540)=8.23 p<0.0001*	'treatment' p<0.0001* 'frequency bin' p<0.0001*
	NREM sleep	'treatment*frequency bin' F(29,540)=9.778 p<0.0001*	'treatment' p<0.0001* 'frequency bin' p<0.0001*
	REM sleep	'treatment*frequency bin' F(29,540)=3.949 p<0.0001*	'treatment' p=0.0061* 'frequency bin' p<0.0001*
Figure 3 (18 months old mice)	Waking	'treatment*frequency bin' F(29,450)=2.42 p<0.0001*	'treatment' p<0.0001* 'frequency bin' p<0.0001*
	NREM sleep	'treatment*frequency bin' F(29,450)=1.173 p=0.248	'treatment' p<0.0001* 'frequency bin' p<0.0001*
	REM sleep	'treatment*frequency bin' F(29,450)=1.79 p=0.008*	'treatment' p<0.0001* 'frequency bin' p<0.0001*
Figure 3 (24 months old mice)	NREM sleep	'treatment*frequency bin' F(29,450)=1.014 p=0.448	'treatment' p<0.0001* 'frequency bin' p<0.0001*
	REM sleep	'treatment*frequency bin' F(29,450)=1.109 p=0.32	'treatment' p<0.0001* 'frequency bin' p<0.0001*
Figure 4 (all groups)	Slow-wave activity (SWA) in NREM sleep	'treatment*time of day*day' F(40,988)=0.03 p>0.99	'treatment' p<0.0001* 'time of day' p=0.124 'day' p=0.377
Figure 4 (Young control mice)	Slow-wave activity (SWA) in NREM sleep	'time of day*day' F(8,89)=11.05 p<0.0001*	'day' p=0.05 'time of day' p=0.93
Figure 4 (Young RW mice)	Slow-wave activity (SWA) in NREM sleep	'time of day*day' F(8,62)=7.443 p<0.0001*	'day' p=0.26 'time of day' p=0.15
Figure 4 (18 months old control mice)	Slow-wave activity (SWA) in NREM sleep	'time of day*day' F(8,63)=5.48 p<0.0001*	'day' p<0.0001* 'time of day' p=0.9995
Figure 4 (18 months old RW mice)	Slow-wave activity (SWA) in NREM sleep	'time of day*day' F(8,72)=14.65 p<0.0001*	'day' p=0.275 'time of day' p=0.69
Figure 4 (24 months old control mice)	Slow-wave activity (SWA) in NREM sleep	'time of day*day' F(8,66)=9.46 p<0.0001*	'day' p<0.0001* 'time of day' p=0.9988
Figure 4 (24 months old RW mice)	Slow-wave activity (SWA) in NREM sleep	'time of day*day' F(8,63)=5.35 p<0.0001*	'day' p=0.82 'time of day' p>0.999

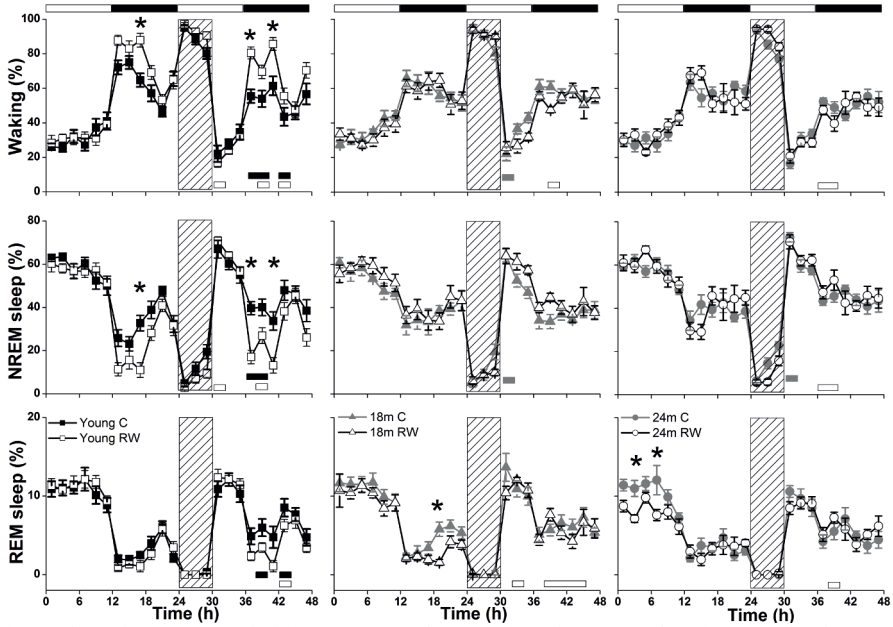

Figure 6.2: Time course of vigilance states, for 24-h baseline (BL), 6-h sleep deprivation (SD, hatched bar) and 18-h recovery for 6, 18 and 24 months old control and running-wheel (RW) mice (left, middle and right panels respectively). Curves connect 2-h mean values (± SEM) of Waking, NREM and REM sleep. The black and white bars above each graph indicate the light-dark cycle. Asterisks at the top of each graph represent significant differences between control and wheel access groups across the 48-h period and bars at the bottom of each graph significant differences between recovery and BL day for each age and condition (post-hoc unpaired and paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVAs, main effects 'treatment', 'time of day', 'day').

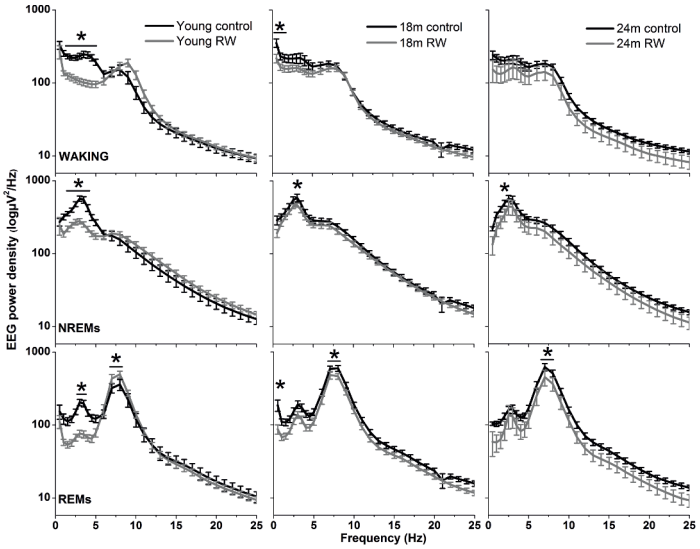

Figure 6.3: Absolute electroencephalographic (EEG) power density in Waking, NREM and REM sleep for 6, 18 and 24 months old control and running-wheel (RW) mice (left, middle and right panels respectively). Asterisks indicate significant differences between the control and wheel access groups (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'treatment', 'frequency bin').

Absolute NREM and REM sleep SWA

To investigate whether the increase in SWA observed in aging could be diminished by the daily use of a running wheel, we computed the time course of absolute NREM sleep SWA in all groups (Fig. 4). We found that EEG SWA in NREM sleep increased as a function of aging, and decreased with the use of the running wheel throughout the 48-h of recordings (Table 1) with interesting differences along the first 12-h of the light period where SWA is more pronounced in NREM sleep [One-way ANOVA, factor 'group', $F(5,66)=90.17$; $p<0.0001$; post-hoc Bonferroni tests: $p<0.05$]. Notably, NREM sleep SWA levels of young control mice did not differ from the 18 months old and 24 months old RW mice (post hoc Bonferroni tests: $p=0.062$ and $p=0.999$ respectively). The two aged control groups and the two aged RW groups differed significantly (post hoc Bonferroni tests: $p=0.045$ and $p=0.018$ respectively). For clarity, we also plot the 24-h average BL EEG SWA in NREM sleep values and 12-h average BL light period REM sleep SWA values next to the EEG SWA in NREM sleep time course. The decrease in absolute SWA in NREM sleep levels in the RW groups, compared to age-matched controls, was the largest in the 24-months old mice (60.44 μV2/0.5Hz), followed by the young mice (47 μV2/0.5Hz) and smallest in the 18-months old mice (24.8 μV2/0.5Hz). In all age groups the decrease was found to be significant [$F(2,33)=82.68$; $p<0.0001$; post-hoc Bonferroni tests: $p<0.0001$]. SD induced alterations in SWA in all groups, particularly an increase was evident in the first 2-6 h of the recovery period (Table 1).

Table 6.2: Mean values (±SEM) in hours of Waking, NREM and REM sleep for 12-h light (L1) and dark (D1) of the baseline (BL) day. Black asterisks indicate significant difference between the group and all the others, and gray asterisk between the same age group (* p≤0.05).

Table 2. 12-h Vigilance states						
Per h	Waking		NREMs		REMs	
Light-Dark	L1	D1	L1	D1	L1	D1
Young RW	3.85 (0.21)	8.92 (0.18) *	6.81 (0.2)	2.78 (0.2) *	1.34 (0.05)	0.3 (0.02)
Young C	3.8 (0.33)	7.62 (0.25)	6.92 (0.23)	4.01 (0.22)	1.28 (0.09)	0.37 (0.04)
18m RW	3.96 (0.27)	7.02 (0.36)	6.79 (0.3)	4.65 (0.35)	1.25 (0.07)	0.34 (0.03)
18m C	4.23 (0.2)	7.08 (0.38)	6.46 (0.23)	4.41 (0.36)	1.31 (0.07)	0.52 (0.05)
24m RW	4.02 (0.15)	6.9 (0.4)	7.03 (0.17)	4.7 (0.35)	0.95 (0.03) *	0.39 (0.07)
24m C	3.87 (0.22)	6.96 (0.33)	6.87 (0.19)	4.64 (0.31)	1.26 (0.06) *	0.39 (0.04)

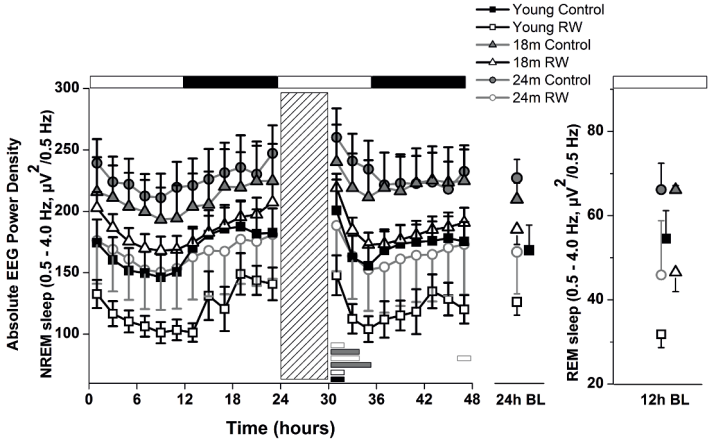

Figure 6.4: Time course of absolute electroencephalographic (EEG) power for the slow-wave activity range (SWA, 0.5-4 Hz) in non-rapid-eye movement (NREM) sleep for 24-h baseline (BL), 6-h sleep deprivation (SD, hatched bar) and 18-h recovery for control and running-wheel (RW) mice (left), 24-h baseline (BL) EEG SWA data in NREM sleep (middle) and 12-h BL EEG SWA in REM sleep (right). EEG SWA in NREM increased as a function of aging, and decreased with the use of a running wheel. Bars at the bottom of each graph significant differences between recovery and BL day for each age and condition (post-hoc Bonferroni multiple comparisons correction t-tests, p<0.05 after significant ANOVA, main effects 'group', 'treatment', 'time of day', 'day').

Table 6.3: Coordinates of the centroids

Table 3			
	x	y	z
Young C	-290.4239343	-351.278	278.2895
Young RW	-258.3819842	-188.679	131.1928
18m C	-480.2993301	-500.791	166.7696
18m RW	-439.3514277	-214.311	326.1029
24m C	-636.4445877	-261.612	322.952
24m RW	-221.3199115	-384.299	412.169

Table 6.4: Euclidian distances between the centroids. Euclidian distances based on the (X, Y, Z coordinates) values in table 3.

Table 4						
	Young C	Young RW	18m C	18m RW	24m C	24m RW
Young C	0.00	221.59	266.16	207.91	360.23	154.24
Young RW	221.59	0.00	384.61	267.20	430.14	344.37
18m C	266.16	384.61	0.00	330.36	325.55	375.32
18m RW	207.91	267.20	330.36	0.00	202.71	289.55
24m C	360.23	430.14	325.55	202.71	0.00	441.97
24m RW	154.24	344.37	375.32	289.55	441.97	0.00

Profiling and classifying slow-wave activity (SWA)

Supervised learning approach
A positive PI was obtained with PI=2.585>0 confirming that SWA in NREM sleep encloses information about age and exercise levels of the mice. Additionally, we could correctly classify mice that were not in the profiling groups (D_p^C) to the corresponding age and exercise group, confirming that the available information can be used to detect previously unknown details of the exercise and age of a mouse. We also computed the success rate of our classification technique which was found to be 100%. This means that we could accurately distinguish between the six groups and assign mice to the appropriate groups, according to their SWA levels, with maximum success rate. By performing dimensionality reduction with PCA we could plot the six different classes in a three dimension (3-D) figure (Fig. 5).
Unsupervised learning approach
In order to detect similarity in SWA data between the different classes, we used Gap statistic clustering to find the optimal number of clusters from the SWA data. The optimal number of clusters was found to be six, indicating that it is equal to the number of classes. The centroids found for each cluster are plotted in Fig. 5 and the coordinates are given in Table 3. The distance between the clusters, as determined by the x, y, z coordinates of their centroids is an indication of similarity in SWA characteristics of the clusters (Table 4). Closer distances to the centroid of the young exercising mice were obtained for young control as well as 18 and 24 months old groups that had a wheel (Euclidean distance: $\|D\|_2$=222,267,344 respectively). Larger distances were found for the 18 and 24 months old control groups (Euclidean distance: $\|D\|_2$=385 and 430 respectively) (Table 4).

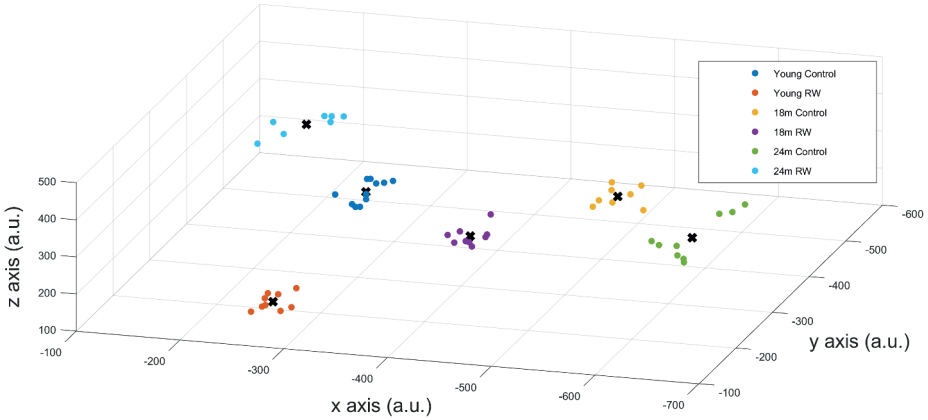

Figure 6.5: X-,y-,z- coordinates of the optimal number of clusters after PCA dimensionality reduction. Scatter plots represent the six experimental groups together with the central points (centroids) (see text for more details).

Discussion

In this study, we show that long-lasting voluntary physical activity in mice, corresponding to regular exercise in humans, alters EEG SWA throughout the whole age spectrum. EEG SWA is implicated in sleep homeostasis and constitutes an important parameter that merits observation. Analytically, our experimental data demonstrate that EEG SWA decreased with exercise and increased with aging. The young mice that were provided with a running wheel had the lowest SWA levels, whereas the oldest sedentary mice had the highest. Notably, the effect of long-term voluntary exercise was sustained for almost two weeks after removal of the running wheel. With regards to sleep architecture, the exercise effect was particularly pronounced in young mice, yet appeared to be attenuated in the older mice. Interestingly, our data show that introduction of moderate exercise even later in life leads concretely to a younger brain phenotype; this is mainly reflected in the NREM sleep EEG SWA. Due to the prominent and expository nature of EEG SWA, we conducted additional analysis using information-theoretic and machine-learning approaches. These methods were able to extract information for age and previous opportunity of physical activity of the mice, and, with cluster analysis, we could classify and accurately distinguish the different groups based solely on their EEG SWA. Thus, our conclusions are three-fold, first, effects of exercise on sleep persist following two weeks after removal of the wheel, second, we suggest that EEG SWA can be used as a physiological marker of brain age in the mouse and, third, we show that exercise can lead to a younger brain phenotype.

NREM sleep EEG SWA: physiological marker of brain age

SWA increases in the course of aging in mice, likely denoting alterations in brain connectivity in the cortex [11]. In the current study, we introduced a running wheel for

daily voluntary use and found that this condition diminishes the age-related increase in EEG SWA towards levels similar to young mice. The data show that EEG SWA in mice increases in the course of aging, regardless of wheel availability, and it decreases in all groups with voluntary exercise. Aged mice that were provided with a wheel and young control mice share very similar SWA characteristics, suggesting that the use of a running wheel alters EEG SWA towards a younger phenotype.

Since distinct characteristic SWA patterns were evident for each group, we conducted additional statistical analysis on EEG SWA data in order to investigate whether SWA can predict the age and previous opportunity of physical activity of the mice. We were able to distinguish groups and successfully predict the age and exercise condition, on the basis of their EEG SWA, with maximum success rate (100%). With cluster analysis based on the Gap statistic approach, we found that the optimal number of clusters was six, and the clusters coincided with the different experimental groups. This indicates that the characteristic features, that can be found in the SWA, by these clustering methods, are sufficiently different to distinguish meaningful groups from the SWA data. The distance between the clusters, as determined by their centroids, is an indication of similarity in SWA characteristics of the clusters.

Physical activity is thought to be beneficial for general health and, additionally in promoting healthy sleep cycles and healthy aging [17, 18, 19, 21], hence, we can consider the young RW mice to be the healthy control group. Therefore, we computed and compared the distances based on the x-, y- and z- coordinates between the young RW group and young, 18, 24 months control and 18 and 24 months RW groups (these are indicated in Table 4). We found that young control mice had the closest distance, whereas aged control mice revealed the largest one. Interestingly, aged RW mice were closer to the young RW mice as compared to aged controls. However, still these distances were larger than the distance between young RW mice and young controls. In addition, as a potential health indicator, we can consider the distance of the other clusters' centroid to the centroid of the young RW mice, as a marker of a "brain age". In other words, we know the chronological age of the mice (18 or 24 months old), and in addition their biological "brain age" which is reduced with physical activity by 30% in 18 months old mice and 20% in 24 months old mice (Fig. 6). In this figure, the distance of the other clusters' centroid to the centroid of the young RW mice, reported earlier in Table 4, is plotted as a 1-D data representation indicating a putative biological "brain age". Thus, our data show that a long-term effect of exercise prevails in the homeostatic component of sleep, i.e. EEG SWA. This modulation is sustained for at least two weeks after exercise has ended. Therefore, we can affirm that the NREM sleep EEG SWA is a physiological marker of brain age in the mouse.

Recent studies demonstrated that SWA in NREM sleep was increased in the course of aging both in the local as well as in the global level [11, 12], however, local cortical neural dynamics and local sleep homeostatic mechanisms, could remain largely unaffected, indicating that protective or compensatory mechanisms may exist to maintain neuronal function stable across the life span [12]. Our study suggests that global dynamics associated with age-induced alterations are mainly affected through regular physical activity leading to a potential 'younger phenotype'.

In addition to a potential ameliorated brain function and/or brain age marker that we propose, SWA may reflect a generally improved physiology due to physical activity.

Since regular exercise can prevent or delay diseases developed throughout life [16], SWA may be a "health marker" of the exercise effect in the course of aging, not only brain-specified but also body-specified. Although, in the present experiment we mainly analyzed a marker of brain activity and can only speculate about bodily health, generally the notion exists that a healthy body likely implies a healthy brain and vice versa, however the pathways in which this may occur remain unknown. In addition thus to a younger brain phenotype, a younger body phenotype may emerge owing to body-brain interactions. Nevertheless, a beneficial effect of exercise is supported by our data displaying consistent results in the whole age spectrum regarding the brain and overall body health.

In contrast to mice, human EEG SWA generally tends to decrease in the course of aging. Following acute, as well as long-term exercise, SWA levels and slow-wave-sleep increased in old humans, reaching levels closer to younger subjects [15, 38, 39, 40]. In old rats, analogously, 8-weeks of daily aerobic exercise led to altered sleep EEG characteristics that reached again levels closer to younger rats. Notably some of the effects were persisting for two weeks after the end of exercise [26]. Our data corroborate this shift towards a younger phenotype due to exercise, i.e. EEG SWA shows an attenuated pattern in aged RW mice. We conjecture that, irrespective of the direction of the SWA change across species, physical activity makes SWA mimic younger patterns. Still, detailed EEG analysis including also SWA levels, that could reveal important insights regarding cortical brain characteristics [41, 42, 43], remains limited across human and animal research and we encourage further studies to solidify this conjecture.

Modest exercise is likely to be beneficial at all life stages, however, it has been proposed that early intervention might be important for a plethora of physiological features, such as cognitive health maintenance and improvement in the course of aging [17, 44]. Our data show that interventions even later in life can also have positive effects on brain activity, such as on SWA levels. This was the case even when the amount of sleep did not change significantly in the aged mice. In elderly humans, the positive effects of physical exercise intervention later in life are evident for periods of weeks to months [45]. For example, it was recently demonstrated that one-year aerobic exercise improved markers of brain function in healthy older adults [21]. Thus, even in the case of later intervention, advantageous effects regarding brain function and consequently overall health are apparent, as shown in our study concerning the aged mice.

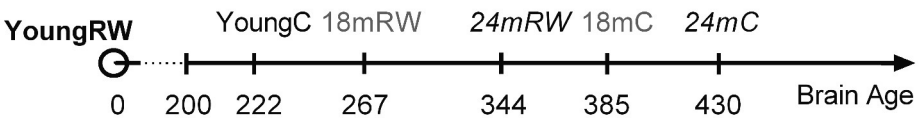

Figure 6.6: Suggested relative brain age of mice represented as the distances between the clusters' centroids of young running-wheel (RW) mice (set as 0, YoungRW) and the other groups. The one-dimension (1-D) values are computed using the Euclidean distances from Table 4 based on the 3-D values reported in Table 3 for young, 18 and 24 months old control and 18 and 24 months RW mice. Hidden brain age is revealed in 18 and 24 months old mice, which is attenuated by 30 and 20% respectively, depicting a rejuvenating path following long-term physical activity.

Wheel activity and behavior

Running wheel activity is depicted as an elective behavior, not only in the laboratory, but also in the wild [46]. It has been associated with enhanced cognitive development across the lifespan and, consequently, protection from age-related cognitive decline [21, 23]. The importance of voluntary wheel-running, where animals are allowed to determine running time, speed, and distance, mimicking human choices, has been discussed earlier [21]. In the current study, we find that with voluntary daily use of a running wheel, the level of EEG SWA can be manipulated in all ages.
It has been suggested that the capacity to exercise is attenuated in the course of aging [47, 48]. However, even low levels of physical activity, including regular daily activities, affect cognitive variables and neuroplasticity as shown in older adult humans [19, 49]. Neurogenesis is known to continue throughout life, but, although its level is attenuated considerably with age [50], physical activity increases cell proliferation rates even in older animals [51]. In a similar way, the aged mice in our study run significantly less compared to young ones; nevertheless, this is sufficient to alter sleep and EEG SWA, indicating the importance of age-matched exercise. We suggest that EEG SWA reveals a rejuvenating path in the aged mice, even with modest exercise levels, as evidenced in the reduced distance between the clusters of the young and/or RW groups of mice compared to aged controls. Previous studies showed that, an age-related deterioration of scale invariance in behavioral rest-activity fluctuations exists, where blocking the running wheel leads to a loss of scale invariance in both young and aged mice [52]. Similar to detrended fluctuation analysis, a mathematical tool, being used to compute the scale invariance, EEG SWA can be defined as a physiological tool to determine brain health in the mouse.

Sustained effects of regular exercise on sleep architecture

The effect of running wheel availability on the sleep-wake cycle in the young mice persisted for at least two weeks after removal of the wheel. It consisted of increased waking and decreased NREM sleep, in the first part of the dark period, resembling the sleep patterns in mice that were recorded concomitantly with the running wheel in their cages [25, 53]. In the course of aging, this effect is diminished. In a similar way, biomarkers of brain activity and neurogenesis, such as BDNF protein levels were shown to be altered due to exercise in young (2 months), middle-aged (15 months) and old (24 months) animals. However, only young animals maintained a significant increase above baseline levels, after two or four weeks after the end of exposure to a running wheel [54, 55, 56], comparable to the changes found in the sleep architecture results in our study, showing that mainly young animals maintained significant sleep-wake alterations. Together, the data suggest that in the course of aging the ability to maintain sleep architectural effects induced by exercise is reduced. In an earlier study with forced exercise in rats [26], sleep architecture was ameliorated after 1-h exercise for eight-weeks, an effect that was sustained even two-weeks after the exercise had ended. In contrast, the mice in our study were not forced but used the running wheel on a voluntary basis, which resulted in moderate levels of total exercise. The voluntary nature may explain why there is an improvement in sleep quality in the aged mice, when the wheel is available and used, and also why this improvement cannot be sustained for very long when the wheel is removed.

Waking and REM sleep EEG power density

The most pronounced effects of exercise on the EEG spectra in all the vigilance states were found in the young mice. The waking spectra showed decreased power in the low frequencies and increased power in the theta frequencies (around 9 Hz) which indicates increased activity and alertness in this group [53, 57]. Interestingly, a decrease in REM sleep slow-wave or delta power and low theta (approximately 0.5-5.5 Hz) and alterations in frequencies between 7-8 Hz were found in all age groups that were housed with a running wheel, compared to their age-matched controls. These EEG frequencies in REM sleep have been associated with hippocampal functioning [58]. The hippocampus is an active region during REM sleep and its function seems to benefit from exercise [39, 59]. Additionally, it has been associated with spatial navigation, particularly in "cognitive map" creations [60, 61] and it is closely associated with the anterior cingulate cortex, a cognitive specific area which is also active during REM sleep [59]. In the elderly, it was demonstrated that exercise could lead to increased connectivity between the hippocampus and the anterior cingulate cortex [62]. Furthermore, wheel running was shown to increase neurogenesis in the hippocampus, and to increase neuronal spine density, synaptic plasticity, neurotrophin levels, and spatial memory function in mice [55, 56]. All these changes may influence REM sleep EEG spectra. Notably, the neuronal effects were considered to be correlated to epigenetic changes in the hippocampus and cerebral cortex and were shown to start within 3-h after wheel running and persist for 2 weeks [55]. Therefore, the consistent decrease of power density in the slow EEG frequencies during REM sleep over all age groups may reflect neuronal changes generated by long-term exercise. However, a difference was found between young and aged groups in frequencies between 7-8 Hz, in which young mice provided with a wheel showed an increase, whereas both aged groups showed a decrease, compared to age-matched controls. It was demonstrated earlier that, despite the fact that the rate of production of new cells in the dentate gyrus of the hippocampus was reduced in aged animals [50], a positive effect of physical activity was apparent on memory performance, neurogenesis and apoptosis [63]. Thus, it seems that the intensity of exercise in combination with the increased age induces different changes in hippocampal brain connectivity evident in these spectra.

Concluding remarks

Here, we show that sleep-wake rhythms and brain EEG variables are improved and that the level of the main sleep homeostatic component is significantly altered with moderate, age-matched exercise. We suggest that SWA is a physiological marker of brain age in the mouse, which emphasizes the importance of regular physical activity to promote and/or maintain physical and mental health. Physical activity, sleep as well as general health, declines in the course of aging. Daily exercise could attenuate the effects of sedentary behaviors, especially in older adults, and it could be prescribed as a first-order "medication" for general body and brain health augmentation. Future research directions could investigate human SWA patterns in order to extract age, exercise or other characteristics and validate the translational aspect of our findings.

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Chapter 7

High-caloric diet and sleep in aged mice

Chronic high-caloric diet accentuates age-induced sleep alterations in mice

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Abstract

Obesity and sleep disturbances comprise major health problems which are likely interrelated. Diet-induced obesity in young mice has been demonstrated to lead towards an altered sleep homeostasis. In the current study, we investigated the effect of chronic (12 weeks) high-caloric diet (HCD, 45% fat) consumption on sleep and the sleep electroencephalogram (EEG) in young and older mice (6-month-old, n=9; 18-month-old, n=8 and 24-month-old, n=4) and compared with age-matched controls on normal chow (n=11, n=9 and n=9 respectively). Half of the 24-month-old mice did not cope well with HCD, therefore this group has a lower n and limited statistical power. We recorded EEG and the electromyogram for continuous 48-h and performed a 6-h sleep deprivation during the second day. In aged HCD fed mice (18 months old) compared to young, an aging effect was still evident, characterized by decreased waking and increased NREM sleep in the dark period, decreased REM sleep during the light period, as well as increased slow-wave-activity (SWA, EEG power in NREM sleep in 0.5-4.0 Hz). Additionally, aged HCD treated mice showed increased NREM sleep and decreased waking, compared to age-matched controls, denoting an enhanced aging phenotype in the sleep architecture. Notably, an overall increase was found in the slow component of SWA (0.5-2.5 Hz) in aged HCD fed mice compared to age-matched controls. Our data suggest that the effect of aging is the dominant variable irrespective of diet. However, a synergistic effect of aging and diet is noted indicating that chronic HCD consumption exacerbates age-associated sleep alterations.

Keywords: obesity, aging, sleep disturbances, slow-wave activity

Introduction

Obesity is nowadays a common characteristic seen across all ages. It raises the risk of several diseases that could lead to premature mortality. For example, the risk of dying from heart disease is increased irrespective of aging, and obesity is thought to underlie age-associated insulin resistance [1, 2].
Obesity and sleep are likely interrelated and may share common pathways [3, 4, 5, 6, 7]. Sleep in people with obesity might appear disturbed and problems falling asleep or waking up often exist. An age-specific prevalence for daytime sleepiness, a frequently reported feature of people with obesity, has been observed [8]. In particular, a linear decline of daytime sleepiness was found in ages from 30 to 75 years old, whereas a higher prevalence of sleepiness was found in people with obesity in ages outside of this limit (<30 and >75 y. o.). In addition to human studies, laboratory animals have been used to study sleep in obesity. Young mice chronically fed with high-caloric diet (HCD), in order to develop obesity, showed sleep architecture alterations [6, 9, 10, 11, 12], slower build-up of sleep pressure and an altered sleep homeostasis [12]. Although the HCD effect on sleep characteristics has received some attention in childhood, adolescent and young adult life, it has not been extensively explored in the course of aging.
Sleep is regulated by two main processes, a circadian process governed by the internal biological clock, and a sleep homeostatic process which is dependent on prior waking and sleep [13, 14]. In mammals, the homeostatic sleep process is thought to be reflected in the NREM sleep electroencephalographic (EEG) slow-wave activity (SWA, EEG power density between 0.75-4.0 Hz) [13, 14].
Aging leads to a redistribution of body fat, with a stabilization or enhancement of visceral fat and fat deposition into tissues such as liver, muscle, and bone [15], similar to the changes seen during HCD consumption [16]. In healthy aged mice, sleep patterns are characterized by increased non-rapid-eye-movement (NREM) sleep as well as increased SWA levels [17, 18, 19, 20, 21, 22, 23]. The effect of HCD as the exclusive diet in aged mice on sleep and the sleep EEG has not been examined, and could give important insights in the effect of diet on sleep in later life.
Therefore, in this study, we investigated the effect of chronic (12 weeks) HCD consumption on sleep architecture and the sleep EEG in young (6 months old) and aged (18 months and 24 months old) C57BL/6J mice during 24-h baseline (BL) recordings, as well as after 6-h of sleep deprivation (SD). We found that the effect of aging on sleep parameters is pronounced irrespective of diet, rendering aging the dominant variable over HCD regarding sleep alterations. Nevertheless, diet and aging likely act synergistically leading to the notion that chronically consumed HCD accentuates age-induced sleep changes.

Materials and methods

Animals

All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Centre (the Netherlands) and were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.
Male C57BL/6JOlaHsd mice of three age groups (6-, 18- and 24-month-old, n=48) (Har-

lan, Horst, the Netherlands) were used for this study. All mice were fed with normal chow (11% fat, 27% protein, 61% carbohydrate, Special Diet Services, UK) before entering the study. Three months before the surgeries (at the age of 3, 15, 21 months for the 6-, 18- and 24-month-old group respectively), mice were partitioned into two groups, the control, in which mice were kept on normal chow diet, and the HCD fed group, in which mice were fed exclusively with high-caloric food, in a pellet form, being very similar to control chow (45% fat mainly derived from lard, 35% carbohydrate, 20% protein; D12451, Research Diet Services, The Netherlands) for 12 weeks. The mice were individually housed under controlled conditions (12:12 h light:dark cycle; lights on at 10:00) with food and water ad libitum in a temperature controlled room (21-22°C).
Originally, the 24-month-old HCD treated group was larger (n=9), however, approximately half of the mice in this age group did not cope well with the HCD that was introduced in their life when they were 21 months old and had severe side effects that led to their premature sacrifice (5 out of 9 mice). Since this did not occur at the age-matched control mice (or any other group used for this study), we attribute the health problems of the aged mice to the altered diet. The remaining 24 months old mice (n=4) did not gain the same amount of weight as the 18 and 6 months old mice that received the same treatment, albeit the amount of food consumed did not seem to differ. Nevertheless, although not delving deeply into the data for this group, we are reporting mainly in the supplementary material and briefly commenting on the obtained results throughout the current study, for research purposes.

Surgeries

On the day of the surgery, 6-month-old HCD fed animals (n=9) weighed on average 47.6 g ($\pm$ 0.8), whereas, their age-matched control animals (n=11) weighed 30.7 g ($\pm$ 0.8), 18-month-old HCD fed animals (n=7) weighed on average 45.43 g ($\pm$ 1.9), whereas their age-matched controls (n=8) weighed 33.5 g ($\pm$ 0.7), and 24-month-old HCD fed animals (n=4) weighed on average 35 g ($\pm$ 2.1), whereas their age-matched controls (n=9) weighed 32.7 g ($\pm$ 1). Under deep anesthesia (Ketamine 100 mg/kg; Xylazine 10 mg/kg; Atropine 1 mg/kg), EEG recording screws (placed above the somatosensory cortex and cerebellum) and electromyogram (EMG) electrodes (placed on the neck muscle) (Plastics One) were implanted as described previously [12, 18, 24]. The wire branches of all electrodes were set in a plastic pedestal (Plastics One, Roanoke, VA) and fixed to the skull with dental cement. The mice were allowed to recover for 7-10 days.

Vigilance state and EEG recordings

The EEG and EMG were recorded with a portable recording system (PS 1 system, Institute of Pharmacology and Toxicology, Zurich, Switzerland) as previously described [12, 18, 24]. Before each recording, a calibration signal was recorded on the EEG and EMG channels. Both signals were amplified, conditioned by analogue filters and sampled at 512 Hz. The signals were filtered through a digital finite impulse response filter and stored with a resolution of 128 Hz. EEG power spectra were computed for 4-s epochs by a FFT routine within the frequency range of 0.25-25.0 Hz.
To record the EEG and EMG, animals were placed into experimental chambers and connected through a flexible cable and a counterbalanced swivel system to the recording

setup. Conditions in the experimental chamber were similar to the home cage, including food and water availability. Before starting the experiment, the animals were allowed to adjust to the experimental conditions for a week. Subsequently, a BL day was recorded, starting at lights on. At the start of the second day, six hours of sleep-deprivation (SD) were conducted. Similar to earlier studies [12, 18, 24, 25], during SD in the experimental chambers, animals were kept awake by gentle handling in which introduction of new food pellets or nesting material and light noises were involved. EEG and EMG were recorded continuously for 48-h, during BL (24-h), SD (6-h), as well as for 18-h of recovery period.

Data analysis and statistics

Three vigilance states (Waking, NREM sleep and REM sleep) were scored offline in 4s epochs by visual inspection of the EEG and EMG signals as well as EEG power density in the slow-wave range, as described previously [12, 18, 24, 25, 26]. For each epoch, the EEG power density in the delta (0.75-4.0 Hz) and theta band (6.25-9.0 Hz) and the integrated EMG value were graphically displayed on a PC monitor to enable scoring of the different vigilance states. The vigilance states were expressed as a percentage of artifact-free recording time. Epochs with artifacts were excluded from the subsequent data analysis of the power spectra, but vigilance states could always be determined. Number of episodes was computed in each vigilance state that were partitioned into ten exponentially increased duration bins from 4s to >1024s similar to [26, 27, 28]. 12-h light and dark (L1, D1, L2, D2 for light and dark periods of the first and second day respectively) mean values of vigilance states were analyzed by two-tailed unpaired t-tests for each period to determine the effect of treatment. Note that, to test the effect of the experimental day, L2 corresponds to the 6-h after SD and is compared to the corresponding 6-h of the BL. Three-way ANOVA was performed to investigate potential alterations on vigilance states, vigilance state episode frequency in duration bins, and 2-h time bins of EEG power density in NREM sleep with main factors 'treatment', 'age', 'day' 'Light-Dark', 'time of day', 'duration bin', 'EEG frequency bin' and '2-h intervals'. Two-way ANOVA was performed regarding the EEG power density of the vigilance states with main factors 'age' and 'EEG frequency bin'. Regarding the Slow/Fast/Overall SWA levels one-way ANOVA was performed with main factor 'treatment'. When appropriate, paired and unpaired post-hoc Bonferroni-corrected student's t-tests were applied to determine the effects of treatment, age and SD. Although novel analysis is included, the 6-month-old group including both high-caloric-diet fed and control mice is the data set published earlier in Panagiotou et al., 2018 [12]. However new analysis on this data set is added here according to the aforementioned tests, that includes the comparisons to the aged groups, the more detailed analysis of the absolute spectra and slow-wave activity as well as the number of vigilance state episodes.

Results

Sleep architecture

HCD and sleep in young and aged mice

Sleep architecture was differentially altered by chronic HCD in young mice (Fig.1 and

Fig.2 left panels) that was elaborately investigated and described previously (data for young mice already published in [12]). HCD mainly induced an increase in REM sleep during the light period without affecting the amount of waking or NREM sleep. By computing the number of episodes, this REM sleep difference was found to be mainly caused by an increase in the short and middle duration REM sleep episodes (8-32s) (post-hoc two-tailed unpaired t-tests, *Supplementary Material* Table S1) (*Supplementary material*, Fig. S1).

18 months old HCD fed mice showed increased NREM sleep during the 24-h BL and decreased waking and no difference in REM sleep compared to their age-matched control mice (Fig. 1, right panel) (18 months old mice: Waking, p=0.028, NREM sleep, p=0.027, REM sleep, p=0.307). The difference in the 18 months old HCD fed mice arises from a decrease in waking and increase in NREM sleep in both 12-h BL light and dark periods (Fig. 1) (Waking, unpaired t-test for L1: p=0.02, D1: p=0.044, L2: p= 0.003 D2: p= 0.013, NREM sleep, unpaired t-test for L1: p=0.0199, D1: p=0.042), L2: p= 0.001, D2: p= 0.012). In contrast, no significant 24- and 12-h effects were found in the 24 months old mice compared to their age-matched controls (*Supplementary material*, Fig. S2A).

The effects of SD were evident in the 18 months old control mice and 24 months control and HCD fed mice (post-hoc two-tailed paired t-tests between BL and after SD time, *Supplementary Material* Table S1) (Fig. 1 and *Supplementary material*, Fig.S2A). Particularly, following SD in the 18 months old control mice, an increase in NREM sleep and decrease in waking was found during the 6-h of the light period, compared to the corresponding 6-h of the BL light period. Additionally, an increase in NREM sleep and decrease in waking was found in both periods following SD in the 24 months old HCD fed mice and 24 months old controls.

The effect of chronic HCD consumption on sleep architecture is also evident in the time course of 2-h values over the day (Fig. 2), where more NREM sleep and less waking is shown in the 18 months old HCD fed mice, effects not evident in the 24 months old age group (*Supplementary material*, Fig.S2B) (post-hoc two-tailed unpaired t-tests, *Supplementary Material* Table S1). As a function of age, effects of SD were more subtle on vigilance states, with 24 months old mice (both control and HCD fed) no longer showing any alterations (p>0.05).

In addition, by computing the number of episodes, it was found that 18 months HCD fed mice had more very short waking episodes (4s) in both BL light and dark periods, and more very short NREM sleep episodes (4s) and less middle (16s) REM sleep episodes in the BL light period as compared to age-matched controls (post-hoc two-tailed unpaired t-tests, *Supplementary Material* Table S1) (*Supplementary material*, Fig. S3). 24 months old HCD fed mice showed more subtle differences compared to age-matched controls having less short (8s) waking episodes and less middle-to-long (128s) NREM sleep episodes in BL light period (post-hoc two-tailed unpaired t-tests, *Supplementary Material* Table S1) (*Supplementary material*, Fig. S4).

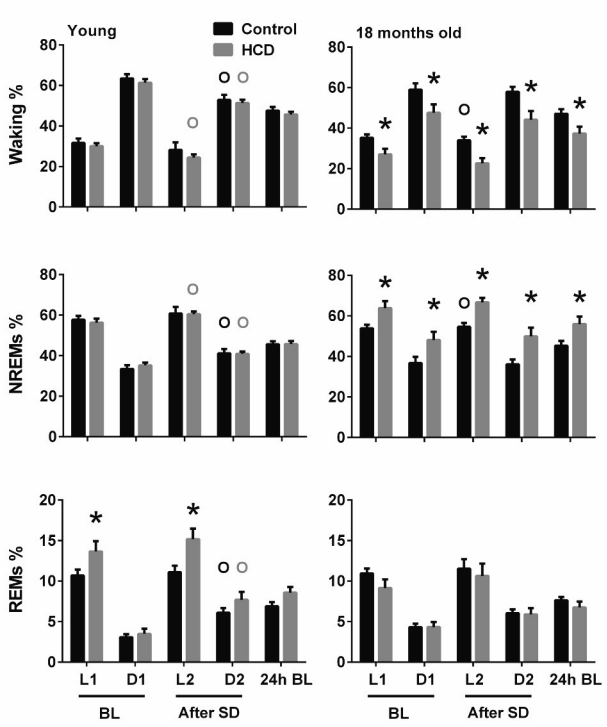

Figure 7.1: Light-dark distribution of each vigilance state (Waking, NREM and REM sleep) during the baseline day (BL: L1, D1) and after sleep deprivation (after SD: L2, D2). Bar plots represent mean ($\pm$ SEM) values (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively) and 24-h values of baseline recordings (24-h BL) for Waking, NREM and REM sleep for control (n=11, n=9 for young and 18 months old mice, black bars) and high-caloric diet fed mice (HCD, n=9 and n=7 for young and 18 months old mice, gray bars). Note that for comparisons between BL and recovery day 6-h of L2 were compared with the corresponding 6-h of the baseline light period. Asterisks indicate significant differences between corresponding age groups (unpaired t-tests for each period, $p<0.05$) and black and gray circles indicate significant differences between recovery and BL day for control and HCD fed mice respectively (post-hoc paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'treatment', 'Light-Dark', 'day').).

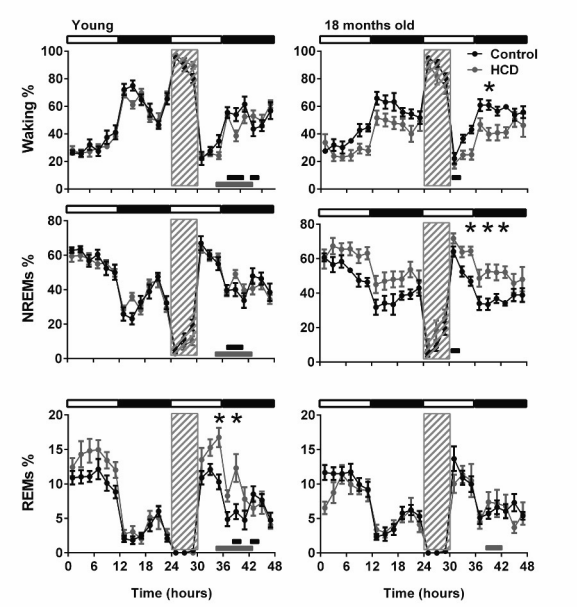

Figure 7.2: Time course of vigilance states (Waking, NREM sleep and REM sleep), for 24-h baseline (BL), 6-h sleep deprivation (SD, hatched bar) and 18-h recovery for young and 18 months old control and high-caloric diet (HCD) treated mice (left and right panels respectively). Curves connect 2-h mean (± SEM) values. The black and white bars above each graph indicate the light-dark cycle. Black asterisks at the top of each graph represent significant differences between the groups across the 48-h period and black and gray bars at the bottom of each graph significant differences between recovery and BL day for each group (post-hoc unpaired and paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVAs, main effects 'treatment', 'time of day', 'day').

Aging and sleep in HCD fed mice

In healthy aged mice, more NREM sleep, stemming from more longer-duration episodes, and less waking during the dark period as well as less REM sleep at the end of the light period has been shown earlier compared to young mice [17, 18, 19, 20, 21, 22, 23]. In the current study, we detected alterations in the sleep architecture of HCD fed mice of the three different age groups, which were mainly found between the young and the aged groups. These consisted of decreased waking during BL dark period, increased NREM sleep in both 12-h BL light and dark periods as well as after SD, and decreased REM sleep in the both light periods, whereas similar data were obtained in the two aged groups for all vigilance states with no significant differences (post-hoc two-tailed unpaired t-tests, *Supplementary Material* Table S1) (Fig. 3A and *Supplementary material*, Fig.S5A).
Regarding the number of episodes, we found significant differences between young and both aged groups of mice that were chronically fed with HCD (post-hoc two-tailed unpaired t-tests, *Supplementary Material* Table S1) (Fig. 3B and *Supplementary material*,

Fig.S5B). During the BL light period, 18 months old HCD fed mice had more brief awakenings (1-4s) and both aged groups had less of the longest category waking episodes (>1024s) compared to young HCD fed mice. Additionally, during the 12-h light period less middle-to-long (64s) and more long (256s) NREM sleep episodes, as well as less middle-to-long (128s) REM sleep episodes were found in the 18 months old HCD fed mice compared to the young animals. During the BL dark period, aged HCD fed mice had more short, middle and long waking and NREM sleep episodes, albeit less very long waking episodes compared to the young HCD fed mice. The number of episodes in waking, NREM and REM sleep did not differ in both 18 months and 24 months old HCD treated when compared to age-matched control mice.

EEG power density (0.5 - 25 Hz)

To study the effect of chronic HCD consumption on the EEG in the course of aging, we calculated the spectral distribution of absolute EEG power density in the frequencies between 0.5 and 25 Hz (post-hoc two-tailed unpaired t-tests, *Supplementary Material* Table S1) (Fig. 4 and Supplementary material, Fig.S5C). Both groups of aged HCD fed mice had higher EEG power density in the slow frequencies (between 0.5-4 Hz in the 18 and 2-7 Hz in the 24 months old mice) during waking, as compared to young HCD fed mice. The NREM sleep EEG spectra in aged HCD fed animals were characterized by a pronounced increase in power density in the slow frequencies (0.5-3 Hz in the 18 and 1-7 Hz in the 24 months old mice) compared to the young HCD fed mice. REM sleep EEG spectra showed higher EEG power density in the 18 months old HCD fed mice in the faster theta frequencies (between 7-8 Hz) and in the 24 months old higher around 2.5 Hz and in the theta frequencies (between 6-9 Hz) compared to young HCD fed mice. Additionally, higher EEG power density in NREM sleep around 3 and 6-7 Hz as well as in REM sleep around 6-8 Hz was noted in the 24 months old compared to 18 months old HCD fed mice. In contrast, EEG power density in young HCD mice tended to be lower in the frequencies below 5 Hz, compared to control. Similar EEG power density spectra were obtained in 18 months and 24 months old HCD fed mice as compared to age-matched controls during waking, NREM and REM sleep. Both aged HCD fed groups showed no significant differences across the vigilance states in the power spectra compared to age-matched controls, albeit a subtle decrease in the REM sleep spectrum in the 18 months old HCD fed mice compared to control around 6 Hz and 22 Hz (post-hoc two-tailed unpaired t-tests, *Supplementary Material* Table S1) (*Supplementary material*, Fig. S6).
As a follow up, we computed the EEG power density in NREM sleep in the frequencies between 0.5 and 25 Hz in 2-h time bins across BL light and dark periods and detected an altered time course in EEG activity between groups, particularly in the slow frequencies (0.5-5 Hz) during the light period (post-hoc two-tailed unpaired t-tests, *Supplementary Material* Table S1) (*Supplementary material*, Fig. S7).

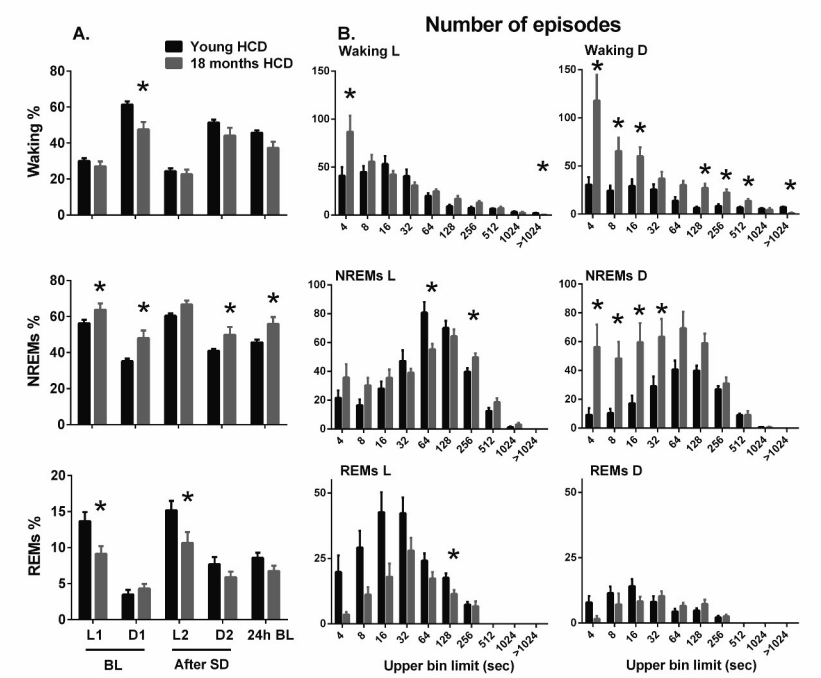

Figure 7.3: Light-dark distribution of each vigilance state (Waking, NREM and REM sleep) during the baseline day (BL: L1, D1) and after sleep deprivation (after SD: L2, D2) and histogram of vigilance state episodes frequency. A. Bar plots represent mean (± SEM) values (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively) and 24-h values of baseline recordings (24-h BL) for Waking, NREM and REM sleep for young and 18 months old high-caloric-diet (HCD) fed mice (n=9 and n=7, black and gray bars respectively). Note that for comparisons between BL and recovery day 6-h of L2 were compared with the corresponding 6-h of the baseline light period. B. Episodes were partitioned in ten exponentially increased duration bins (upper bin limit from 4 to >1024s) in baseline light and dark periods. Asterisks indicate significant differences from the young HCD fed mice (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'age', 'Light-Dark', 'day').

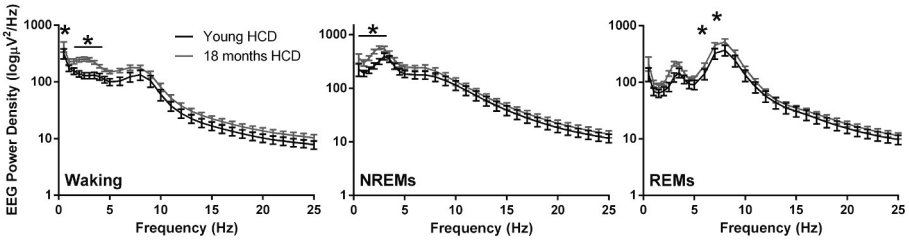

Figure 7.4: Electroencephalographic (EEG) power density in Waking, NREM and REM sleep for young and 18 months old high-caloric-diet (HCD) fed mice (log $\mu V^2/Hz$, 0.5-25 Hz). Black asterisks indicate significant differences between 18 months old and young HCD fed mice (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, p<0.05 after significant ANOVA, main effects 'age', 'EEG frequency bin').

Slow-wave activity in NREM sleep (SWA)

The spectral data suggested that HCD specifically affects EEG SWA during NREM sleep. To investigate this further, we computed the absolute NREM SWA levels in all groups in 2-h intervals across the 48-h of recordings (Fig. 5 and *Supplementary material*, Fig.S2C) (*Supplementary Material* Table S1). We found that the effect of HCD differs across aging, where 18 months old HCD fed mice showed no alterations, 24 months old HCD fed mice showed an increase and young HCD mice an overall decrease in SWA levels compared to their age-matched controls (One-way ANOVA, factor 'treatment', p<0.0001; post-hoc Bonferroni t-tests: p<0.05). Interestingly, the three HCD fed groups significantly differed from each other (post-hoc Bonferroni tests: p<0.0001) showing an increase in SWA as a function of age.

Additionally, taking into account the results from figure S7, where significant differences in the slow frequency range, as well as an altered behavior between slow (0.5-2.5 Hz) and fast (2.5-4Hz) slow-waves [29] were detected, we computed the time course of slow and fast SWA for all groups (Fig. 5). An overall significant treatment effect was noted in the slow and fast SWA range (One-way ANOVA, factor 'treatment', Slow SWA: p<0.0001, Fast SWA: p<0.0001). Post-hoc multiple comparisons across treatment groups revealed that the 18 months old HCD fed mice had increased slow SWA levels, whereas 24 months old HCD fed mice showed an increase in both the slow and fast SWA, and therefore an overall increase, compared to age-matched controls. In contrast, the decrease in overall SWA seen in young HCD mice emerged from a decrease of the slow SWA, whereas no change was found in the fast SWA. Therefore, slow and fast slow-waves are differentially affected by age and diet.

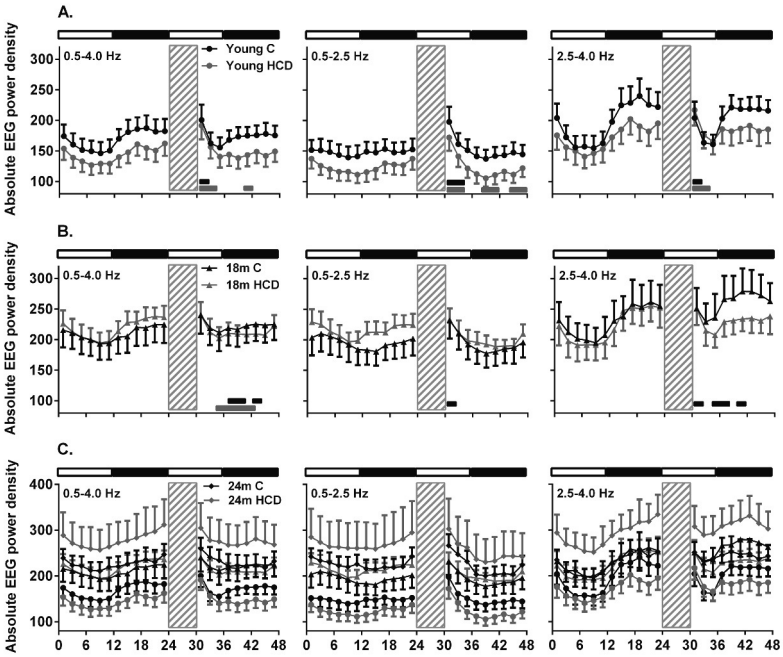

Figure 7.5: Time course of absolute electroencephalographic (EEG) power density ($\mu V^2/Hz/0.5Hz$) for the overall slow-wave activity range (SWA, 0.5-4 Hz) in non-rapid-eye movement (NREM) sleep (left graph), for the slow SWA 0.5-2.5 Hz (middle graph) and the fast SWA 2.5-4 Hz (right graph) for 24-h baseline (BL), 6-h sleep deprivation (SD, hatched bar) and 18-h recovery for young (A), 18 months old (B) and 24 months old (C) control and high-caloric diet fed (HCD) mice. Bars at the bottom of each graph indicate significant differences between recovery and BL day for each age group (post-hoc paired t-tests with Bonferroni multiple comparisons correction p<0.05, after significant ANOVAs, main effects 'day' and 'time of day'). Overall decreased EEG SWA levels were revealed in the young HCD fed mice (0.5-4 Hz and 0.5-2.5 Hz) and overall increased levels in 18 months (0.5-2.5 Hz) and 24 months old HCD fed mice (0.5-4 Hz, 0.5-2.5 Hz and 2.5-4Hz) compared to control mice (one-way ANOVA, factor 'treatment'; p<0.05).

Discussion

In this study, the effect of chronic HCD consumption was investigated in the course of aging. Both aged HCD fed groups demonstrated similar sleep architectural patterns that significantly differed from the young HCD fed mice, consisting of increased NREM sleep, decreased waking and decreased REM sleep, as well as absence of very long waking episodes. Additionally, higher EEG power density during BL recordings was obtained in the slow frequencies of all vigilance states in both aged HCD fed mice compared to young HCD fed mice. Compared to age-matched controls, 18 months old HCD fed mice showed increased NREM sleep and decreased waking in both light and dark periods, whereas 24 months old HCD fed mice showed no alterations. Notably, the slow and

fast SWA component was differently affected in the course of aging in HCD fed mice. Decreased levels of the slow SWA were found in the young HCD fed mice, whereas increased levels were found in the 18 months old HCD fed mice, while for the 24 months old a general increase in both slow and fast SWA was found. Our data show that aging is the dominant variable inducing alterations in the sleep architecture and the sleep EEG. However, the combined effect of aging plus diet seems to provoke detrimental effects leading to exacerbated aging patterns in sleep and the sleep EEG.

Sleep architecture

Recent research showed that chronic HCD consumption in young mice leads to altered sleep homeostasis with a slower build-up rate of sleep pressure, increased REM sleep during the light period, an increase in consecutive NREM-REM sleep cycles and changes in waking, NREM, and REM sleep episode number and duration throughout the 24-h [6, 9, 10, 11, 12]. During natural aging, mice demonstrate increased NREM sleep during the dark period emerging from an increase in long NREM sleep and short waking episodes, and decreased REM sleep during the light period [17, 18, 19, 20, 21, 22, 23]. In accordance, aged mice show augmented SWA levels [17, 18]. Similar effects were noted in both aged HCD groups in the current study compared to the young HCD fed group, where aging characteristics were evident in the sleep architecture, including increased NREM sleep, decreased waking and decreased REM sleep, as it is shown earlier [17, 18, 19, 20, 21, 22, 23]. However, compared to age-matched controls, 18 months old HCD fed mice demonstrated an accentuated aging phenotype in sleep architecture, characterized by a further increase in NREM sleep and decrease in waking.
Total fat mass is known to peak at middle age in both humans and rodents [15]. In addition, older people have elevated levels of abdominal adipose tissue compared to young individuals [15, 30, 31], which has been characterized as a risk factor for several diseases. Adipose tissue from agouti mice, which are genetically obese, showed features of premature aging, higher levels of reactive-oxygen-species (ROS) and DNA damage [32]. In our study, the notion is developed that obesity and aging may act synergistically, affecting sleep architecture. This may occur through the increasing total fat mass levels as well as adipose tissue likely induced by both obesity and aging in the 18 months old mice, which coincide to 50-60 y.o. in humans, intensifying this way the already age-induced sleep architectural characteristics.
Insufficient orexin signaling has been implicated in several medical conditions including obesity [3], and neurodegenerative diseases [33, 34]. Being characterized as a regulator of vigilance states, energy homeostasis, as well as the reward system and feeding [35, 36], orexin may be implicated in the regulation of sleep in young HCD fed mice, as increased levels of REM sleep and an increased number of NREM-REM sleep cycles were found [12], similar to what occurs in narcolepsy [37]. In the course of aging, a decline in the orexin system has been demonstrated in many species, including mice [38, 39], rendering orexin an important factor underlying age-related metabolic and sleep/wake cycle alterations. We suggest that chronic HCD may influence the orexin system, however, this effect is expressed differently in the sleep of young and aged mice, since aging can act as a synergistic factor to obesity.
Furthermore, adenosine kinase has been found to be increased in the cerebral cortex of

old animals [40]. Under a high-fat diet, it has also been shown that levels of adenosine monophosphate-activated protein kinase (mAMPK) decline [41]. In addition, blocking adenosine kinase is likely to prolong sleep and further increase SWA in the EEG [42]. Although the specific mechanisms are not known, it seems that the adenosinergic system could play a role in the way HCD affects sleep architecture and general sleep function in the course of aging.

EEG power density and slow-wave activity

When compared to the young HCD fed mice, aged HCD fed mice demonstrate alterations in the waking, NREM and REM sleep spectra, in accordance with the aging sleep and EEG phenotype. In particular an increase in EEG SWA in NREM sleep was observed in the course of aging. These changes are consistent with a previous study, suggesting that sleep pressure is increased in aged compared to young mice [18]. The question remains whether HCD in aging adds further effects.
We therefore computed the changes in the EEG power density spectra between 0.25-25.0 Hz in 2-h time bins (*Supplementary Material*, Fig. S7), and found the most pronounced differences between the groups between 0.5-5 Hz. In addition, during NREM sleep we observed differences in the time course of the slow (0.5-2.5 Hz) and fast (2.5-4.0 Hz) frequency bands within the slow-wave range. It is known that SWA can be partitioned in two different bands according to a different origin of the waves, first the slow component, with frequencies around 0.5-2.5 Hz which correspond to cortico-cortical connections, and second the faster frequencies within 2.5-4 Hz which correspond to thalamo-cortical connections [29]. Therefore, we investigated the time course of the overall SWA (0.5-4 Hz), as well as of the slow and fast frequency ranges for each group (Fig. 5). The data show increased SWA levels in aged mice irrespective of diet, confirming earlier findings from our group [18]. This shows that aging is the prevailing parameter influencing SWA. Interestingly, it seems that HCD acts synergistically with aging differently affecting the slow and fast component of SWA. In the case of the 18 months old mice, the slow component of SWA showed an overall increase under influence of HCD, whereas the fast component remained unaltered, leading to no significant change in the overall SWA levels. In contrast, 24 months old HCD fed mice showed an increase in both the slow and fast component which resulted in an overall increase in SWA. The increase in the slow component of SWA in 18 months old HCD fed mice and the increase in both the slow and fast components in the 24 months old HCD fed mice indicating an amplification of the aging phenotype by HCD. In addition, it is important to note that young and aged mice respond differently to chronically consumed HCD as reflected in the SWA levels. We show that the slow component increased in both aged groups fed HCD, whereas young HCD fed mice had lower SWA levels, particularly in the slow SWA range.
Brain aging is still unavoidable, however, a line can be drawn between successful brain aging which is important for increased longevity and health, and pathological brain aging, which can accelerate health deterioration. Recently, it was shown that, during healthy aging, in mice slow-wave characteristics alter, probably reflecting brain connectivity changes [18]. It has also been proposed that during normal brain aging imbalances of neurotransmitters and increases in inflammatory signaling and oxidative stress may occur [15]. High-fat diet has been demonstrated to promote insulin resistance and

metabolic dysfunction in both humans and rodents, however the impact on brain function remains poorly understood [6, 43, 44, 45, 46]. It has been proposed that HCD could impede neuronal plasticity and modulate astrocytic and microglial signaling [47, 48, 49]. Additionally, it has been hypothesized that the slow SWA component, emerging from the cortico-cortical connections in the brain, involves glial activities [50, 51]. Theories have been developed linking astrocytes, metabolism and sleep via mechanisms such as the neuronal-glial 'lactate shuttle', according to which synaptic glutamate release triggers lactate production and release in astrocytes, evident in brain lactate levels, that increase during wakefulness and decrease during NREM sleep in parallel with NREM SWA [48, 52, 53, 54, 55]. In the current study, we show that particularly the slow component of SWA was altered under influence of HCD in aged mice, intensifying the aging effects. Therefore, the data suggest that HCD may impact cortical brain connectivity through a glial pathway. In contrast, the thalamocortical pathway underlying the fast SWA component is clearly influenced by aging, however, it is differently affected by HCD across the age groups. The thalamus and its associated network may play a different role in the interaction between HCD and aging. Compared to young controls, hypocretin receptor 2 levels were found to be declined in the thalamus of 24 months old mice but not in 18 months old [38]. We therefore suggest that alterations in fast SWA are the result of an interacting influence of HCD and aging on thalamo-cortical activity.

The changes caused by HCD in mice resemble metabolic disturbances observed in humans, however, HCD consumption can also promote novel metabolic perturbations, as well as accelerate individual aspects of age-related metabolic disturbances [15, 56]. In our results, it seems that altered brain connectivity, apparent in the EEG SWA, is likely synergistically impacted by age and diet.

Furthermore, chronic HCD consumption may play an important role in regulating age-related increases in oxidative stress. It was demonstrated that lipoprotein metabolism and oxidant/antioxidant status were altered in obesity, irrespective of age. Nevertheless, obesity-related lipid and lipoprotein alterations were attenuated, whereas, oxidative stress was worsened in older adults [57]. In aged HCD-induced obese mice, HCD exacerbated age-related oxidative stress in the brain via increased NADPH oxidase (NOX), rendering NOX activity a player in the ability of HCD to detrimentally affect brain homeostasis in aged individuals, likely associated with feed-forward cascades of oxidative stress and brain inflammation [58]. In the current study, we find that chronic HCD consumption amplifies aging effects on the slow component of NREM sleep SWA levels of aged mice. Recent studies implicate aberrant or excessive activation of microglia in the pathogenesis of brain aging, which could also account for the augmented levels found when aging is combined with HCD. Further research is undoubtedly needed regarding the relation of glial activation and associated pathways with chronic obesity and brain wave alterations in order to verify our aforementioned hypotheses.

A limitation of the present study is the unsuccessful chronic HCD treatment of the 24 month old group. Approximately 50% of the mice long-term fed with HCD were euthanized because of serious health problems (5 out of 9). C57BL/6 mice are known to express a multifactorial age-related susceptibility to plasma lipid levels, after dietary intake of high fat, rendering this strain susceptible to lipid-related vascular complications such as atherosclerosis [59, 60]. Additionally, an age-dependent change in hepatic lipid and antioxidant metabolism inducing increased susceptibility to lipid peroxidation has

been noted [61, 62]. Therefore, chronic HCD, starting above 18 months of age, is likely to lead to severe health problems as seen in the present study. Therefore, the results and conclusions from the 24 months old group should be treated with caution as the number of animals included is too low for a proper statistical analysis.

Concluding Remarks

It has already been established that the deterioration of structure and function of organs in aging is associated with oxidative stress, genetic instability and disruption of homeostatic pathways [63]. Obesity has been associated with a wide range of chronic diseases that could potentially lead to premature mortality. There are similarities in metabolic dysregulation in aging and obesity, and, thus, it is likely that these conditions share similar pathways. In addition, the brain comprises one of the most crucial sites at the intersection of age and metabolic dysfunction, since cognitive dysfunction and increased risk of dementia constitute debilitating deficits. In our study, we show that, HCD influences sleep differently in the course of aging. Although aging is the prevailing parameter, sleep architecture and the sleep EEG are also affected when HCD is introduced, indicating that the combination of chronic HCD and increased age impacts sleep and brain health. Notably, the slow SWA levels were altered in all age groups under HCD indicating general brain connectivity changes due to this diet, albeit in opposite directions in young and aged mice. Taking the opposite direction effect into account, as well as the attenuated effect of prolonged waking on subsequent EEG SWA in NREM sleep in the young HCD fed mice compared to controls [12], we can conclude that: short-term benefits emerge by consuming HCD at a young age, lowering the burden of the sleep debt incurred, however with increasing age this effect is diminished and faster brain aging is induced instead.

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Competing financial interests

The authors declare no conflict of interest.

Data Accessibility Statement

The authors deposit supporting information and datafiles to Figshare for data archiving https://figshare.com/s/b1a96e3601b05c86d423

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Supplementary Material

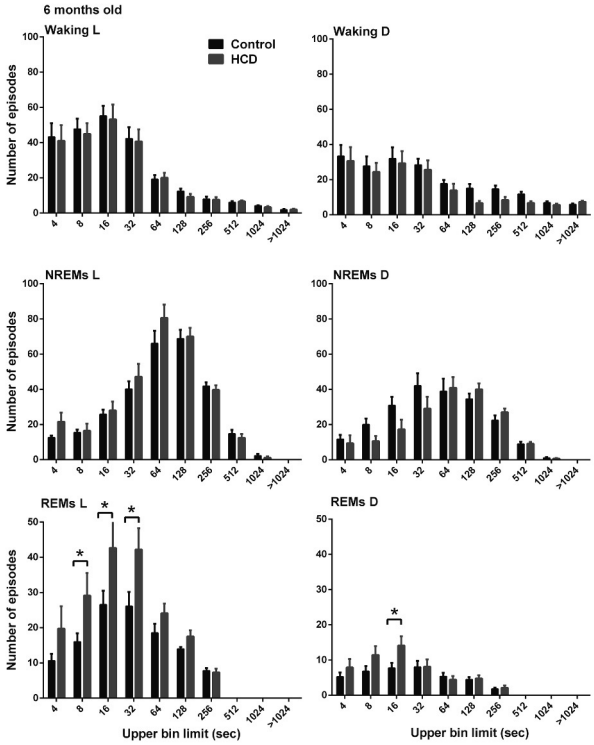

Figure 7.1: Histogram of vigilance state episodes frequency during the baseline light (L) and dark (D) period. Episodes were partitioned in ten exponentially increased duration bins (upper bin limit from 4 to >1024s) for young control high-caloric-diet (HCD) fed mice in baseline light and dark periods. Asterisks indicate significant differences between the groups (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'treatment', 'Light-Dark', 'duration bin').

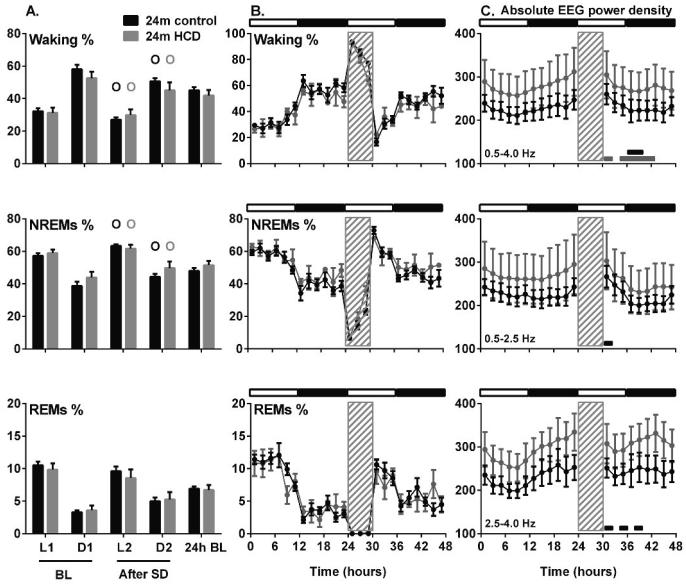

Figure 7.2: A. Light-dark distribution of each vigilance state (Waking, NREM and REM sleep) during the baseline day (BL: L1, D1) and after sleep deprivation (after SD: L2, D2). Bar plots represent mean ($\pm$ SEM) values (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively) and 24-h values of baseline recordings (24-h BL) for Waking, NREM and REM sleep for control and high-caloric diet fed mice (HCD) (n=9 black bars and n=4 gray bars respectively). B. Time course of vigilance states (Waking, NREM sleep and REM sleep), for 24-h baseline (BL), 6-h sleep deprivation (SD, hatched bar) and 18-h recovery. Curves connect 2-h mean ($\pm$ SEM) values. The black and white bars above each graph indicate the light-dark cycle. No differences were found between groups (p>0.05). C. Time course of absolute electroencephalographic (EEG) power density ($\mu V^2/0.5Hz$) for the overall slow-wave activity range (SWA, 0.5-4 Hz) in non-rapid-eye movement (NREM) sleep, for the slow SWA 0.5-2.5 Hz and the fast SWA 2.5-4 Hz for 24-h baseline (BL), 6-h sleep deprivation (SD, hatched bar) and 18-h recovery. Black and grey bars represent differences for baseline and after sleep deprivation days.

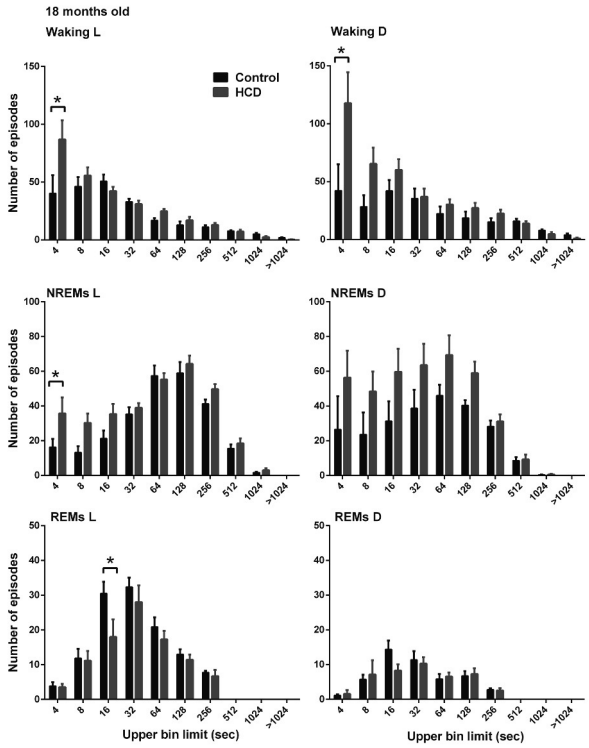

Figure 7.3: Histogram of vigilance state episodes frequency during the baseline light (L) and dark (D) period. Episodes were partitioned in ten exponentially increased duration bins (upper bin limit from 4 to >1024s) for 18 months old control high-caloric-diet (HCD) fed mice in baseline light and dark periods. Asterisks indicate significant differences between the groups (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'treatment', 'Light-Dark', 'duration bin').

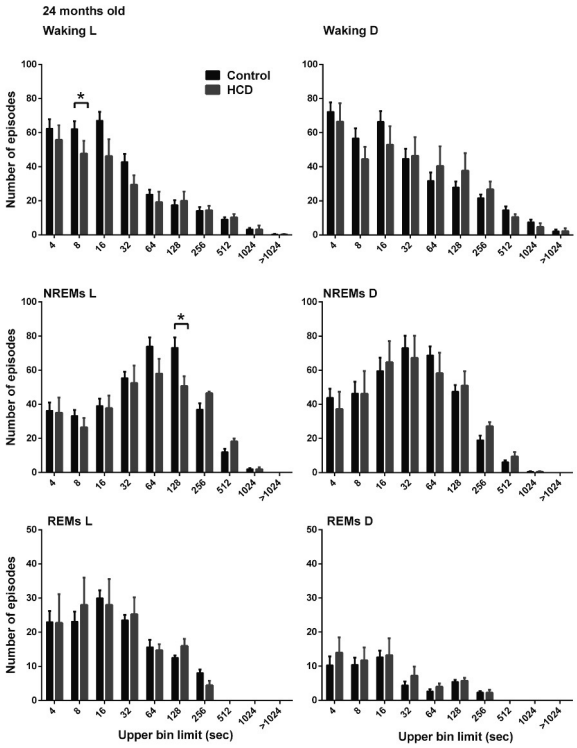

Figure 7.4: Histogram of vigilance state episodes frequency during the baseline light (L) and dark (D) period. Episodes were partitioned in ten exponentially increased duration bins (upper bin limit from 4 to >1024s) for 24 months old control high-caloric-diet (HCD) fed mice in baseline light and dark periods. Asterisks indicate significant differences between the groups (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'treatment', 'Light-Dark', 'duration bin').

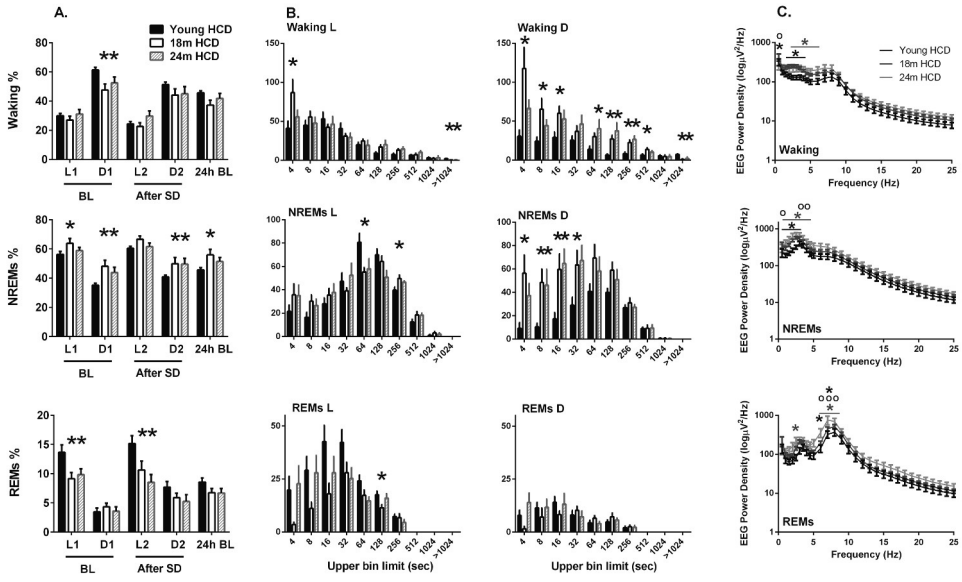

Figure 7.5: A. Light-dark distribution of each vigilance state (Waking, NREM and REM sleep) during the baseline day (BL: L1, D1) and after sleep deprivation (after SD: L2, D2). Bar plots represent mean ($\pm$ SEM) values (L1, D1, D2 correspond to 12-h values and L2 to 6-h values for the recovery period after SD, for light and dark periods during the 48-h recordings respectively) and 24-h values of baseline recordings (24-h BL) for Waking, NREM and REM sleep for all high-caloric-diet fed (HCD) groups. B. Histogram of vigilance state episodes frequency during the baseline light (L) and dark (D) period. Episodes were partitioned in ten exponentially increased duration bins (upper bin limit from 4 to >1024s) for young, 18 and 24 months high-caloric-diet (HCD) fed mice in baseline light and dark periods. Asterisks represent differences between groups (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'age', 'Light-Dark', 'day'). C. Electroencephalographic (EEG) power density in Waking, NREM and REM sleep for young, 18 months and 24 months old high-caloric-diet (HCD) fed mice (log $\mu V^2/Hz$, 0.5-25 Hz). Black and gray asterisks indicate significant differences of 18 months and 24 months old respectively compared to young HCD fed mice and black circles significant differences between 18 months and 24 months old HCD fed mice (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'age', 'EEG frequency bin').

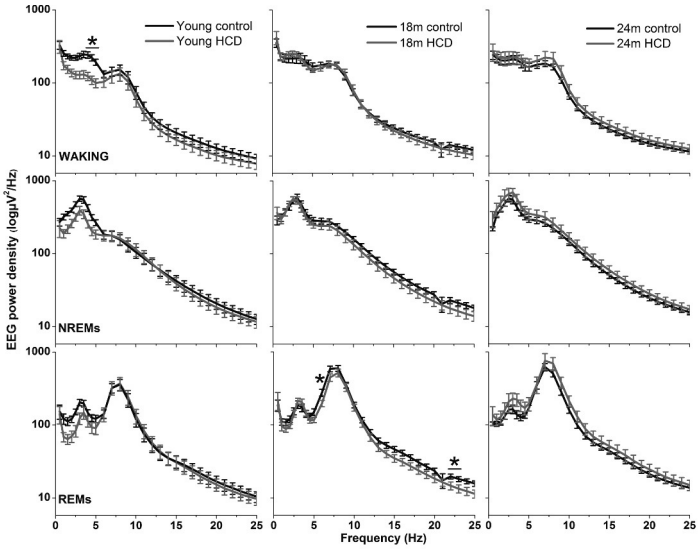

Figure 7.6: Electroencephalographic (EEG) power density in Waking, NREM and REM sleep for young, 18 months and 24 months old control and high-caloric-diet (HCD) fed mice (log $\mu V^2/Hz$, 0.5-25 Hz). Asterisks indicate significant differences between control and HCD fed groups (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'treatment', 'EEG frequency bin').

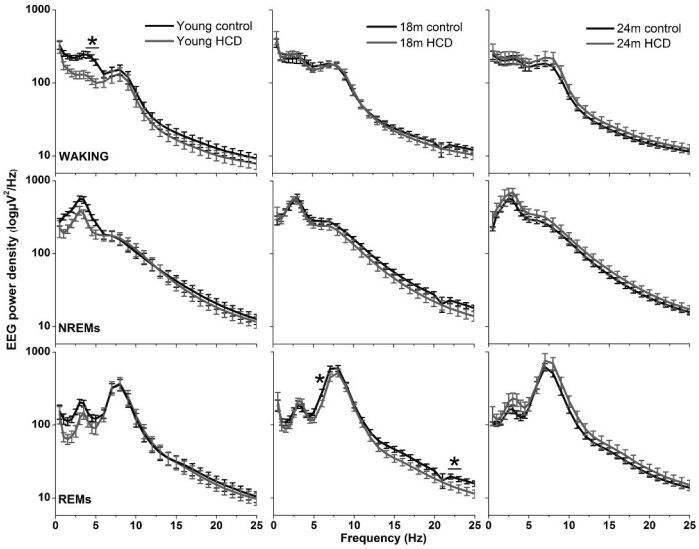

Figure 7.7: Electroencephalographic (EEG) power density in NREM sleep (expressed as %12-h values of the light period) in the frequencies between 0.5 and 25 Hz in 2-h time bins across the BL light period for young (green line), 18 months (dark gray line) and 24 months (light gray line) old high-caloric-diet (HCD) fed mice and young controls (black line). Black and gray asterisks indicate significant differences of 18 months and 24 months old HCD fed compared to young HCD fed mice respectively, black circles significant differences between 18 months and 24 months old HCD fed mice and black lines on the top of each graph indicate significant differences between young control and young HCD fed mice (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'age', 'EEG frequency bin', '2-h intervals').

Chapter 8

Sleep and chronic dim-light-at-night in aging

Effects of chronic dim-light-at-night exposure on sleep in young and aged mice

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Abstract

Dim-light-at-night (DLAN) exposure is associated with health problems, such as metabolic disruptions, immunological modulations, oxidative stress, sleep problems, and altered circadian timing. Neurophysiological parameters, including sleep patterns, are altered in the course of aging in a similar way. Here, we investigated the effect of chronic (three months) DLAN exposure (12L:12Dim-light, 75:5lux) on sleep and the sleep electroencephalogram (EEG), and rest-activity behavior in young (6-month-old, n=9) and aged (18- n=8, 24-month-old, n=6) C57BL/6J mice and compared with age-matched controls (n=11, n=9 and n=8, respectively). We recorded the EEG and electromyogram continuously for 48-h and conducted a 6-h sleep-deprivation. A delay in the phase angle of entrainment of locomotor activity and daily vigilance state rhythms was apparent in mice following DLAN exposure, throughout the whole age spectrum, rendering sleep characteristics similar among the three age DLAN groups and significantly different from the age-matched controls. Notably, slow-wave-activity in NREM sleep (SWA, EEG power density in 0.5-4.0 Hz) was differentially altered in young and aged DLAN mice. Particularly, SWA increased as a function of age, which was further accentuated following DLAN exposure. However, this was not found in the young DLAN animals, which were characterized by the lowest SWA levels. Concluding, long-term DLAN exposure induced more pronounced alterations in the sleep architecture of young mice, towards an aging phenotype, while it enhanced age-associated sleep changes in the older groups. Our data suggest that irrespective of age, chronic DLAN exposure deteriorates sleep behavior and may consequently impact general health.

Keywords: sleep, dim-light-at-night (DLAN), aging, electroencephalogram, sleep deprivation, slow-wave-activity

Introduction

The invention of electrical light in the past century has entirely reformed modern society. Although generally it is considered an everyday convenience, artificial light exposure particularly at night, has been linked with health problems such as metabolic, immunological, and behavioral rhythm disturbances across a wide age spectrum [1]. One such rhythm vulnerable to perturbation, is the sleep-wake cycle [2, 3, 4].

Sleep is mainly regulated by two processes, a circadian process, which is controlled by the biological clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus and a sleep homeostatic process, which is dependent on prior waking and sleep [6, 7]. In mammals, the homeostatic sleep process is considered to be reflected in the NREM sleep electroencephalographic (EEG) slow-wave activity (SWA, EEG power density between 0.75-4.0 Hz) [6, 7]. Sleep deprivation has been experimentally used to test the sleep homeostatic process and investigate sleep characteristics such as sleep architecture and the sleep EEG under elevated sleep pressure conditions [7].

Concomitant to a multitude of physiological parameters that are affected owing to aging, sleep does not remain unaltered. Aging in laboratory animals, such as mice, is characterized by increased NREM sleep during the dark active period, emerging from an increase in longer NREM sleep episodes, as well as increased EEG SWA levels [8, 9, 10, 11, 12, 13, 14]. In addition to increased EEG SWA, the overall morphology of slow-waves has been found to be modulated, likely indicating changes in connectivity of cortical brain areas in the course of aging [8, 9].

The combination of aging and long-term light-at-night exposure may have additional effects on sleep and the sleep EEG. Whole-night light exposure (40 lux) in young human subjects shows acute effects on sleep architecture and brain activity [15]. In particular, shallow sleep, frequent arousals, reduction of activity in the slow-wave and spindle frequency bands during NREM sleep and theta frequency bands during REM sleep have been found. In a similar group of subjects, dim-light-at-night (DLAN) exposure (5 lux) has been shown to affect sleep quality, increasing waking after sleep onset, NREM sleep stage 1 and REM sleep, whereas decreasing NREM sleep stage 2 [16]. In animals, disruption of molecular circadian rhythms and weight gain in mice [17], disturbance of the daily sleep-wake cycle in the rat [18], as well as altered daily rhythms and behavioral patterns in the grey mouse lemur [19] have been demonstrated following DLAN exposure. Although detrimental effects of DLAN exposure, particularly in the long-term, have been suggested, the impact in aging on sleep is not documented yet and is important as both the aging population and light exposure have increased in the last decades.

In the current study, the effect of long-term DLAN exposure (5 lux) on behavior, sleep architecture and the sleep EEG in aged mice is investigated. 18- and 24-month old mice are exposed to three consecutive months of DLAN and the effects are compared to young 6-month old mice exposed to the same treatment as well as to age-matched controls exposed to a light-dark cycle. A delay in the phase angle of entrainment of daily vigilance state rhythms was noted in mice across the whole age spectrum and sleep characteristics between young and aged mice shared many similarities following DLAN exposure. Additionally, sleep changes already seen in aged mice, such as the attenuated 24-h vigilance state rhythm amplitude, the increase in NREM sleep particularly during the dark period, as well as elevated SWA levels, were further augmented in aged mice after DLAN expo-

sure. The data indicate that although the most pronounced effects are found at a young age, chronic DLAN exposure is detrimental as far as the sleep process is concerned, irrespective of age.

Materials and methods

Animals

Male C57BL/6JOlaHsd mice of three age groups (6, 18 and 24 months old, n=51) (Harlan, Horst, the Netherlands) were used for the current study. All mice were exposed to light:dark conditions [(12:12 h, 75lux:0lux), with 0 lux corresponding to complete darkness (1lux=1 lm/m2)]. Three months before the surgeries mice were partitioned into two groups [at the age of 3 months for the 6 months old group (n=11 and n=9 for control and DLAN exposed), at the age of 15 months for the 18 months old group (n=8 and n=8 for control and DLAN exposed) and at the age of 21 months for the 24 months old group (n=9 and n=6 for control and DLAN exposed)]. Mice were individually housed under controlled conditions [12:12 h light:dark cycle (75lux:0lux) (Control group) or 12:12 h light:DLAN (75lux:5lux) (DLAN group); lights on at 10:00AM] with food and water ad libitum in a temperature controlled room (21-22 °C). Light in all conditions was emitted by white fluorescent tubes placed above the cage. Light intensities were verified by AvaSpec 2048-SPU (Avantes BV, the Netherlands) light meter.
All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Centre (the Netherlands) and were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Surgeries

Under deep anesthesia (Ketamine 100 mg/kg; Xylazine 10 mg/kg; Atropine 1 mg/kg), EEG recording screws (placed above the somatosensory cortex and cerebellum) and electromyogram (EMG) electrodes (placed on the neck muscle) (Plastics One) were implanted as described previously [9, 21]. The wire branches of all electrodes were set in a plastic pedestal (Plastics One, Roanoke, VA) which was fixed to the skull with dental cement. The mice were allowed to recover for 7-10 days.

Light schedules and behavioral recordings

The mice were kept on a 12:12 h Light:Dark schedule. In order to investigate the effect of long-term DLAN exposure on behavior, vigilance states and the EEG, the mice were exposed for 12 weeks to 12:12h Light:DLAN. Subsequently EEG and EMG electrodes were implanted and sleep recordings were performed. Locomotor activity for the light-dark schedule (15 days) and the three-month DLAN condition was monitored by passive infrared detectors (PIR, Actimetrics software, Wilmette, Il, USA) prior to the EEG/EMG recordings. The rhythm period and strength of the 24-h component was determined by F-Periodogram analysis, a method generally designed to detect rhythmic components in rest-activity data, similar to previous studies [18, 20]. The strength of the 24-h component particularly is defined as the difference between the peak and the 99% confidence

limit in individual periodograms of rest-activity data, an approach elaborately described earlier to quantify the magnitude of the 24-h component [20].

EEG recordings

The EEG and EMG were recorded with a portable recording system (PS 1 system, Institute of Pharmacology and Toxicology, Zurich, Switzerland) as previously described [9, 21]. Before each recording, a calibration signal (10 Hz sine wave 300 μV peak-to-peak) was recorded on the EEG and EMG channels. Both signals were amplified, conditioned by analogue filters and sampled at 512 Hz. The signals were filtered through a digital finite impulse response filter and stored with a resolution of 128 Hz. EEG power spectra were computed for 4-s epochs by a FFT routine within the frequency range of 0.25-25.0 Hz. To record the EEG and EMG, animals were placed into experimental chambers and connected through a flexible cable and a counterbalanced swivel system to the recording setup. Conditions in the experimental chamber were similar to the home cage, including light conditions, food and water availability. Before starting each experiment, animals were allowed to adjust to the experimental conditions for a week. Subsequently, a baseline (BL, 24h) day was recorded, starting at lights on. Subsequently, a second day was recorded at the start of which, six hours of sleep-deprivation were conducted by gentle handling [9, 21, 22]. EEG and EMG were recorded continuously during BL and sleep deprivation, as well as for 18 hours of recovery period. In total, continuous 48h were recorded including the BL, the sleep deprivation, and recovery period.

Data analysis and statistics

Three vigilance states (Waking, NREM sleep and REM sleep) were scored offline in 4s epochs by visual inspection of the EEG and EMG signals, as described previously [9, 21, 22]. For each epoch, the EEG power density in the slow-wave (delta activity, 0.75-4.0 Hz) and faster frequency range (theta activity, 6.25-9.0 Hz) and the integrated EMG value were graphically displayed on a PC monitor to enable scoring of the different vigilance states. The vigilance states were expressed as a percentage of artifact-free recording time. Epochs with artifacts were excluded from the subsequent data analysis of the power spectra, but vigilance states could always be determined. In general, we tested for and found distributions and equal variances in the sampled distributions and therefore parametric tests were used. To test whether DLAN or age altered the daily vigilance state distribution, a one-way ANOVA was performed on 24-h mean values (factor 'treatment', 'age') and three-way ANOVA was performed on 12-h and 6-h and 2-h mean values of vigilance states (factors 'treatment', 'age', 'Light-Dark', 'time of day', 'day'). Note that L2 corresponds to 6-h value of light period following sleep deprivation and is compared to the corresponding 6-h value of BL. Additionally, to test potential differences in SWA in NREM sleep, a one-way with factor 'group' and a three-way ANOVA with factors 'treatment', 'time of day', 'day' for all groups was performed, as well as a two-way ANOVA with factors 'time of day' and 'day' for each group to test potential alterations due to sleep deprivation. A two-way ANOVA on the 24-h EEG power density values of the three vigilance states with main factors 'treatment' and 'EEG frequency bins' was performed. Cumulative NREM and REM sleep plots were calculated by subtracting the minutes of sleep during deprivation and recovery from the corresponding baseline value

and summing the difference with the preceding hour and were tested through a two-way ANOVA with factors 'treatment', 'age' and 'time of day'. Additionally, linear regressions of the accumulated NREM sleep and REM sleep regained during the 18-h recovery period were computed and slope differences of the linear regression were tested with unpaired t-tests. To test the strength of the behavioral rhythm, a one-way ANOVA was performed with factor 'strength' across groups. When appropriate, paired and unpaired post-hoc Bonferroni-corrected student's t-tests were applied to determine the effects of treatment and sleep deprivation.

Results

Rest-activity behavioral data

Examples of representative rest-activity behavior from animals exposed to LD and DLAN conditions of the three different age groups are shown in Figure 1 (15 days). By performing F-periodogram analysis, we computed the rhythm period which revealed one peak in all mice not significantly different from 24 hours (Mean±SD, Young LD: 24 $\pm$ 0.0, Young DLAN: 24.08 $\pm$ 0.21, 18m LD: 24.02 $\pm$ 0.05, 18m DLAN: 23.97 $\pm$ 0.25, 24m LD: 24.01 $\pm$ 0.072, 24m DLAN: 24.51 $\pm$ 0.48). In addition, the strength of the behavioral rhythm was computed (Mean$\pm$SD, Young LD: 0.21 $\pm$ 0.02, Young DLAN: 0.11 $\pm$ 0.03, 18m LD: 0.12 $\pm$ 0.03, 18m DLAN: 0.09 $\pm$ 0.03, 24m LD: 0.15 $\pm$ 0.03, 24m DLAN: 0.12 $\pm$ 0.04) and was found decreased in all DLAN mice as compared to age-matched controls with significant results in young and 18 months old mice ($p<0.05$) as well as in both 18 and 24 months old control groups compared to the young control group but across DLAN mice (One-way ANOVA, factor 'strength' $p=0.0001$, post-hoc unpaired t-tests between groups).

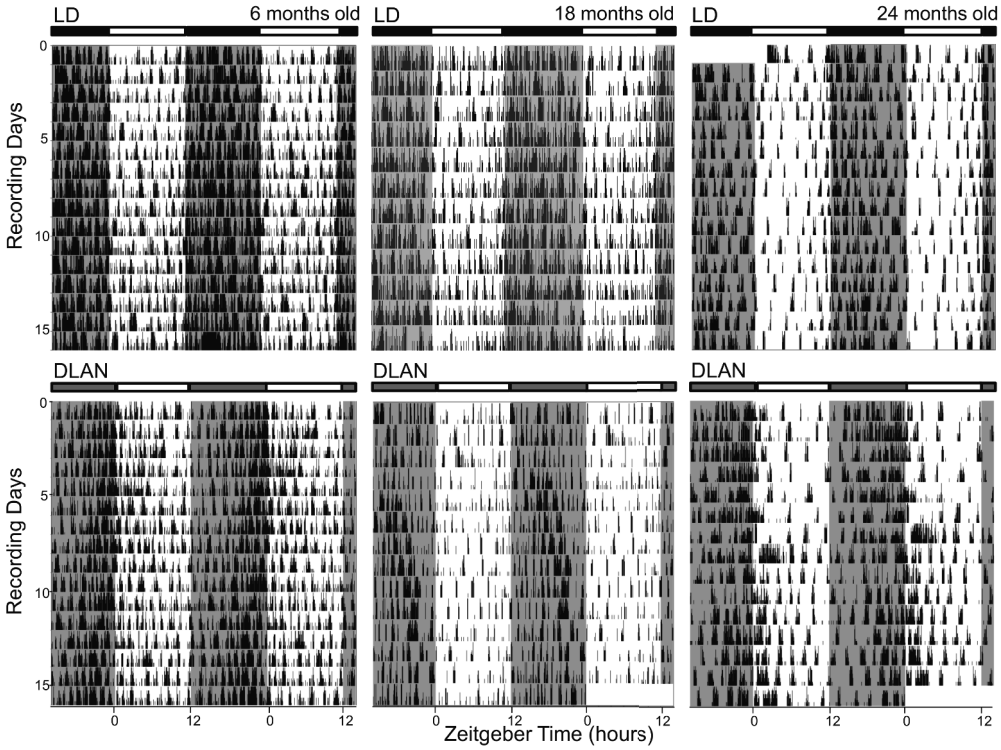

Figure 8.1: Representative double-plotted actograms of 3 mice of three different age groups (6, 18, 24 months old) exposed to control 12:12h light:dark (top, LD, 75lux:0lux) (15 days) and 12:12h light:dim-light-at-night (bottom, DLAN, 75lux:5lux) (15 days). Note the concentration of activity to the later part of the dim period in the DLAN group.

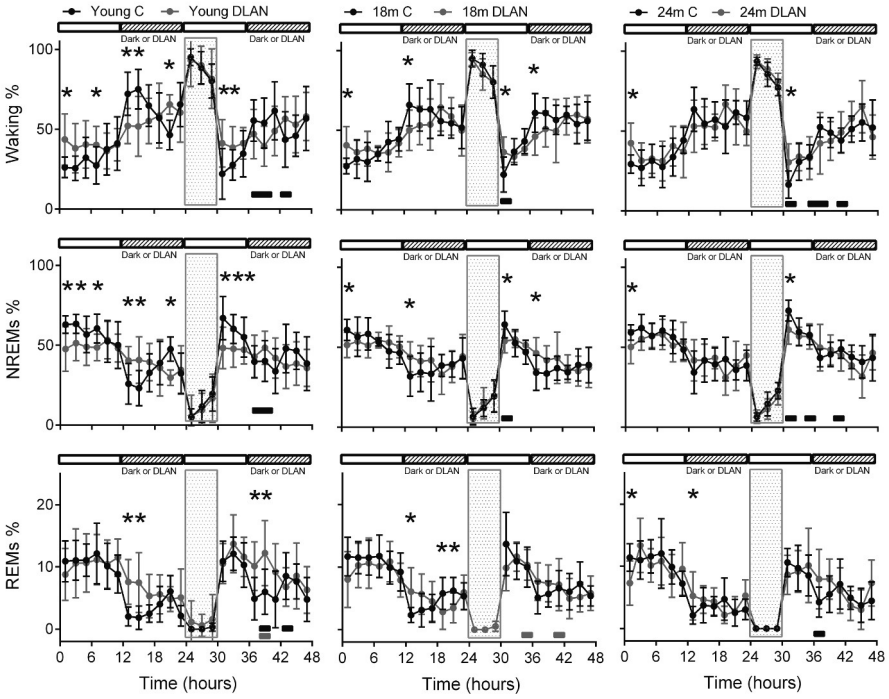

Figure 8.2: Time course of vigilance states, for 24-h baseline, 6-h sleep-deprivation (hatched bar) and 18-h recovery period for young, 18 and 24 months old mice in either control light:dark conditions (n=11, 8, 9 respectively) or following 3 months exposure to dim-light-at-night (DLAN) conditions (n=9, 8, 6 respectively). Curves connect 2-h values of waking, NREM and REM sleep (Mean $\pm$ SD). The bars above each graph indicate the light-dark/dim light cycle. Black asterisks at the top of each graph represent significant differences between the control and the DLAN mice for each age group across the 48-h period, while bars at the bottom represent differences between baseline day and recovery day (post-hoc unpaired and paired t-tests with Bonferroni multiple comparisons correction when appropriate, $p<0.05$ after significant ANOVAs, main effects 'treatment', 'time of day', 'day').

Vigilance states: Waking, NREM and REM sleep amount

The largest differences regarding the effects of DLAN on the amount of vigilance states were found in the young DLAN mice compared to age-matched controls. In particular, increased wakefulness was found in young DLAN in the light period and decreased wakefulness during the dim light/dark period, as well as more REM sleep in the dim light/dark period (Figure 2) (post-hoc unpaired t-tests Waking: $F(11,432)=1.5$; $p=0.13$, NREM: $F(11,432)=1.63$; $p=0.088$ and REM sleep: $F(11,432)=0.69$; $p=0.747$ with significant interactions 'treatment*time of day' $p<0.0001$, Appendix, Table A.1), with more REM sleep apparent in the 12-h dim light/dark period and more waking in the light period following sleep deprivation (Figure 3) (post-hoc unpaired t-tests Waking: $F(1,72) = 0.0001$; $p=0.986$, NREM sleep: $F(1,72) = 0.034$; $p=0.855$ and REM sleep: $F(1,72) =$

0.353; p=0.554 with significant interactions 'treatment* Light-Dark' p<0.05, Appendix, Table A.1). Notably, all 6, 18 and 24 months old DLAN mice showed a delay in the increase in waking at the beginning of the dim light period and a similar delay in the increase in NREM sleep at the start of the light period, compared to age-matched controls (post-hoc unpaired t-tests, 18 months old, Waking: F(11,336)=0.954; p=0.489, NREM sleep: F(11,336)=0.859; p=0.581 and REM sleep: F(11,336)=0.672; p=0.765 and 24 months old, Waking: F(11,306)=0.84; p=0.6, NREM sleep: F(11,306)=0.894; p=0.547 and REM sleep: F(11,306)=0.642; p=0.792 with significant interactions 'treatment*time of day' p<0.05, Appendix, Table A.1) inducing in turn an attenuated 24-h vigilance state rhythm amplitude (defined as the vigilance state difference between light and dark periods). These effects were not only apparent during BL, but were also consistent in the recovery period following sleep deprivation. Additionally, compared to BL, sleep deprivation did not induce any alterations in the amounts of waking and NREM sleep in all DLAN mice, contrary to changes induced in the age-matched control mice; a subtle increase was only evident in REM sleep in the dim light period after sleep deprivation in young and 18 months old DLAN mice compared to BL (post-hoc paired t-tests between BL and after sleep deprivation period values, following significant interactions 'treatment*time of day' and main factor 'day' p<0.05, Appendix, Table A.1).

24-h and 12-h values did not differ in 18 and 24 months old mice long-term exposed to DLAN compared to age-matched controls (p>0.05) (Figure 3). Compared to BL, young, 18 and 24 months old DLAN mice had more REM sleep in the dim light period following sleep deprivation (post-hoc paired t-tests between BL and after sleep deprivation period values, following significant interactions 'treatment*Light-Dark' and main factor 'day' p<0.05, Appendix, Table A.1). In contrast, young, 18 and 24 months old control animals showed more alterations in their vigilance state distribution after sleep deprivation, including more NREM and REM sleep and less waking in the dark period in the young mice, less waking and more NREM sleep in the dark period in 18 months old mice, and less waking and more NREM sleep in both light and dark periods in 24 months old mice (post-hoc paired t-tests, between BL and after sleep deprivation period values, following significant interactions 'treatment*Light-Dark' and main factor 'day' p<0.05, Appendix, Table A.1).

Mice exposed to long-term DLAN showed a gradual increase as a function of age in NREM sleep, particularly evident in the 6-h light period after sleep deprivation as well as decreased REM sleep in the 12-h dim light period after sleep deprivation, with significant differences between young and 24 months old mice (Figure 4) (post-hoc unpaired t-tests, NREM sleep: F(2,88)=0.6; p=0.55, with significant interaction 'age*Light-Dark' and main factors p<0.05, REM sleep: F(2,88)=0.593; p=0.555 with main factors 'Light-Dark' and 'day' p<0.05, Appendix, Table A.1). 24-h BL values did not differ between the young mice long-term exposed to DLAN and both 18 and 24 months old (p>0.05). Interestingly, waking, NREM and REM sleep 2-h values did not differ among the three DLAN age groups (p>0.05) in the BL day, with only small decreases in REM sleep during the second day following sleep deprivation, in the oldest age groups compared to the young (Figure 5) (post-hoc unpaired and paired t-tests, F (22,480) = 0.366; p=0.997, with main factors 'treatment', 'time of day' and 'day' <0.0001, Appendix, Table A.1).

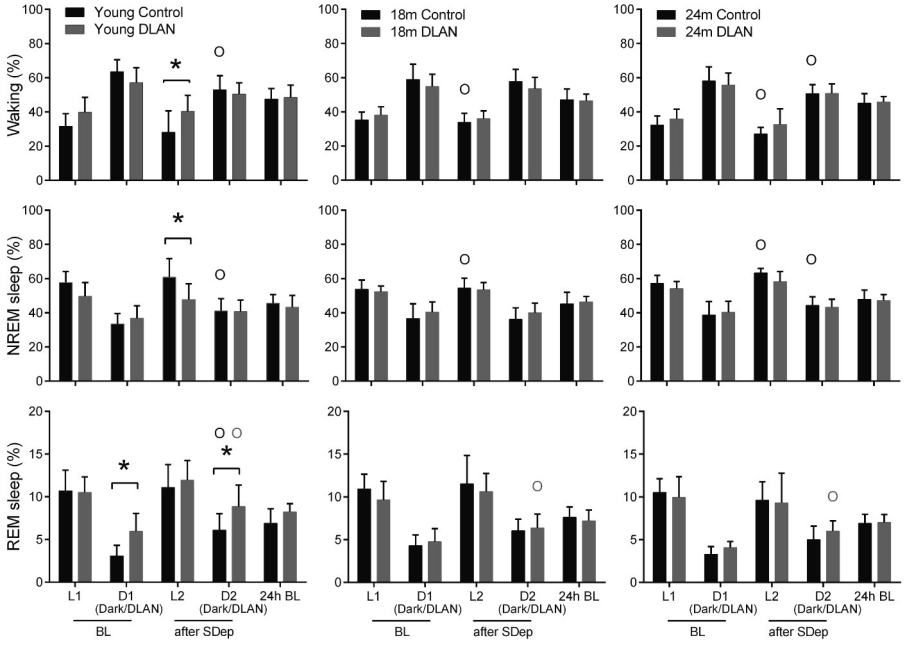

Figure 8.3: Distribution of each behavioral state (Waking, NREM and REM sleep) during the baseline day and after sleep deprivation. Bars represent mean ($\pm$ SD) values (L1, D1 (data during baseline (BL)), D2 correspond to 12-h values and L2 (L2,D2: data after sleep deprivation) to 6-h values for the recovery period after sleep deprivation, for light and dark/dim light periods during the 48-h recordings respectively) and 24-h values of baseline recordings (24-h BL) for Waking, NREM and REM sleep for the three age groups control light:dark conditions (n=11, 8, 9 for young, 18 and 24 months old) or following three months exposure to dim-light-at-night (DLAN) conditions (n=9, 8, 6 respectively). Asterisks indicate significant differences between the groups and circles indicate significant differences between recovery and baseline day (post-hoc unpaired and paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVAs, main effects 'treatment', 'Light-Dark', 'day').

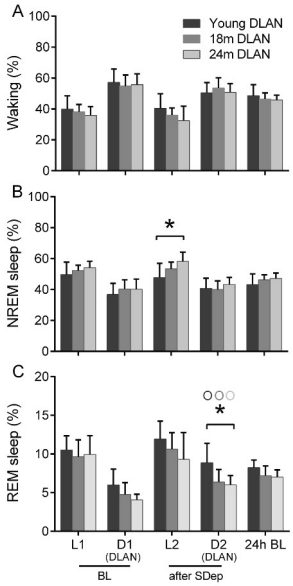

Figure 8.4: Distribution of each behavioral state (Waking, NREM and REM sleep) during the baseline day and after sleep deprivation in the age groups long-term exposed to dim-light-at-night (DLAN). Bars represent mean (± SD) values (L1, D1 (data during baseline (BL)), D2 correspond to 12-h values and L2 (L2, D2: data after sleep deprivation) to 6-h values for the recovery period after sleep deprivation, for light and dark/dim light periods during the 48-h recordings respectively) and 24-h values of baseline recordings (24-h BL) for Waking (A), NREM sleep (B) and REM sleep (C) for the three age DLAN groups (young: n=9; 18 and 24 months old: n=8 and 6). D1 and D2 correspond to dim light periods. Asterisks indicate significant differences between the age groups and circles indicate significant differences between recovery and baseline day for the same group, shown in gradually decreasing gray colors (post-hoc unpaired and paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'age', 'Light-Dark', 'day').

Additionally, we computed the cumulative NREM and REM sleep lost or gained (Figure 6) (post-hoc unpaired t-tests, $F(46,480)=0.34$; $p>0.99$ with main factors 'age' $p<0.0001$, 'time of day' $p<0.0001$). Although a significant part of NREM sleep lost was not regained in all groups, DLAN or aging and their combination led to less NREM sleep regained, as compared to young control mice ($p<0.05$). The slopes of the regained NREM sleep differed significantly between young control and young DLAN mice (Rate of accumulated NREMs percentage/hour, young control: 7.908 ± 1.127; young DLAN: 2.816 ± 1.553; $p=0.007$) and between the three age control groups (young control: 7.908 ± 1.127; 18 months control: 0.5108 ± 1.335; 24 months control: 3.843 ± 1.155; $p<0.0001$). Interestingly, young DLAN mice showed similar patterns to aged DLAN mice ($p>0.05$).
Contrary to NREM sleep, REM sleep lost was almost fully regained in young DLAN mice showing an increase during recovery similar to young controls, whereas aged con-

trol and DLAN mice were not able to regain the lost REM sleep showing a less steep increase (post-hoc unpaired t-tests, Appendix, Table A.1) with slopes significantly different between age control groups (young control: 2.78 $\pm$ 0.29; 18 months control: 1.819 $\pm$ 0.32; 24 months control: 1.397 $\pm$ 0.22; p=0.0012) a trend between age DLAN groups (young DLAN: 2.752 $\pm$ 0.43; 18 months DLAN: 1.853 $\pm$ 0.32; 24 months DLAN: 1.66 $\pm$ 0.27; p=0.07576).

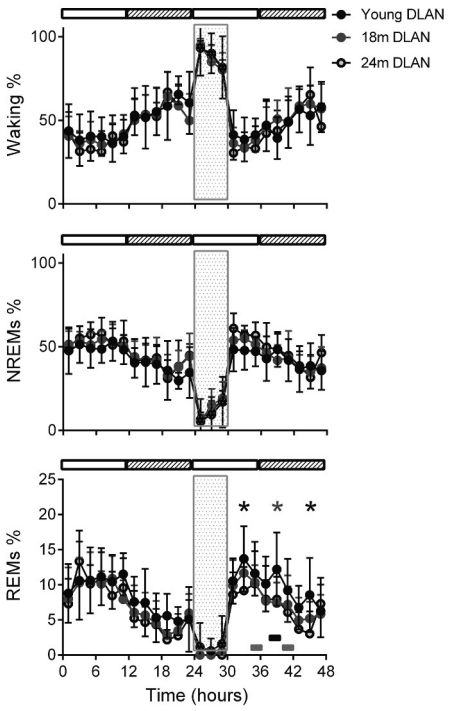

Figure 8.5: Time course of vigilance states, for 24-h baseline, 6-h sleep-deprivation (hatched bar) and 18-h recovery period for young, 18 and 24 months old mice following 3 months exposure to dim-light-at-night (DLAN) conditions (n=9, 8, 6 respectively). Curves connect 2-h values of waking, NREM and REM sleep (Mean $\pm$ SD). The bars above each graph indicate the light-dim light cycle. The gray and black asterisks indicate significant differences between the young and the 18 and 24 months old mice respectively (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction when appropriate, p<0.05 after significant ANOVA, main effects 'treatment', 'time of day', 'day').

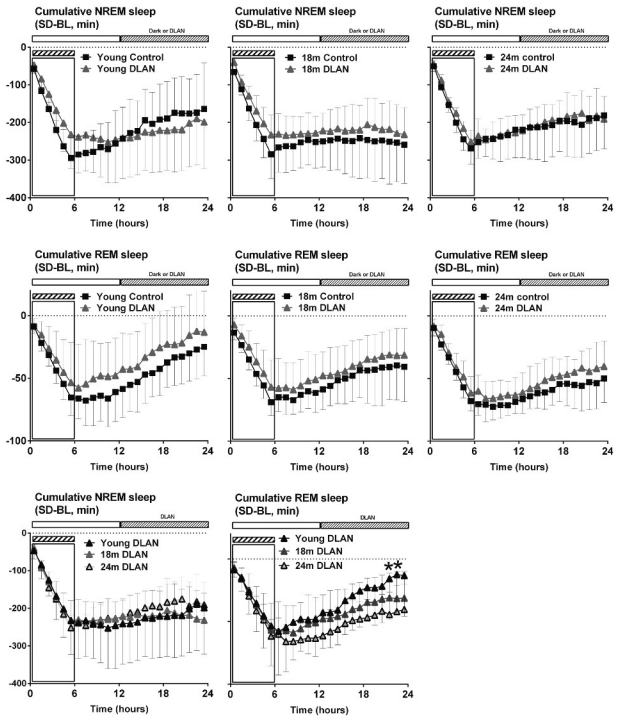

Figure 8.6: Time course of accumulated NREM sleep and REM sleep lost and regained during the 6-h sleep deprivation and the 18-h recovery period for young, 18 and 24 months old mice in either control light:dark (n=11, 8, 9 respectively) or following three months exposure to light:dim-light-at-night (DLAN) conditions (n=9, 8, 6 respectively). Curves connect 1-h values that are calculated by subtracting the minutes of sleep during deprivation and recovery from the corresponding baseline value and summing the difference with the preceding hour. Asterisks indicate significant differences between young DLAN and 24 months DLAN mice (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'treatment'/'age', 'time of day'). The slopes of the regained NREM sleep differed significantly between young control and young DLAN mice and between the three age control groups (*more details in the Results section of the text*).

EEG power density between 0.5-25 Hz and slow-wave activity in NREM sleep

The most pronounced differences regarding the EEG power spectra were found in the young mice groups (Figure 7). Young DLAN mice showed a decrease in the slow frequencies in the waking (1-5 Hz), and NREM sleep (1-4.5 Hz) spectrum (post-hoc unpaired t-tests, Waking: $F(29,540)=4.9$; $p<0.0001$, NREM sleep: $F(29,540)=5.93$; $p<0.0001$, Appendix, Table A.1). In the REM sleep spectrum, DLAN exposure decreased power in the slow wave and theta frequencies (3-3.5 and 8 Hz) (post-hoc unpaired t-tests, REM sleep: $F(29,540)=1.348$; $p=0.1082$ with main factors 'treatment' and 'EEG frequency bins' $p<0.0001$, Appendix, Table A.1). In contrast, DLAN exposure did not induce any alterations in the waking, NREM and REM sleep spectra of the 18 months old mice

($p>0.05$). In the 24 months old mice, however, long-term DLAN exposure induced alterations in the opposite direction compared to the young mice, with increased activity in the slow waves in waking and NREM sleep (0.5 and 3.5 Hz in waking, and 2.5-3.5 in NREM sleep), as well as increased theta activity during REM sleep (7-8 Hz) (post-hoc unpaired t-tests, Waking: $F(29,390)=1.5$; $p=0.0557$, NREM sleep: $F(29,390)=2.2$; $p=0.0006$, REM sleep: $F(29,390)=1.15$; $p=0.2786$, with main factors 'treatment' and 'EEG frequency bins' $p<0.0001$, Appendix, Table A.1).

SWA in NREM sleep was differentially affected in young and aged DLAN mice as compared to their age-matched controls. A characteristic increase in SWA was apparent as a function of age, as previously observed [8, 9]. Chronic DLAN exposure induced an overall further increase in the 24 months old mice in the levels of SWA across the 48-h recording time (Figure 8) ($F(55,1033)=0.093$; $p>0.99$, with main factors 'treatment' $p<0.0001$ 'time of day' $p=0.057$, 'day' $p=0.006$, Appendix, Table A.1). In contrast, a decrease in the levels of SWA across the 48-h was found following chronic DLAN exposure in the young mice. SWA in NREM sleep was significantly different across the 48-h among groups, except for the 18 months old control, 18 months old DLAN and 24 months old control mice which shared similar values (One-way ANOVA, factor 'group', $p<0.0001$; post-hoc t-tests between groups in the overall period, $p<0.05$). Following sleep deprivation, an increase in SWA was evident in all groups (post-hoc paired t-tests, Appendix, Table A.1). This increase lasted between 2-6 h in the light period following sleep deprivation.

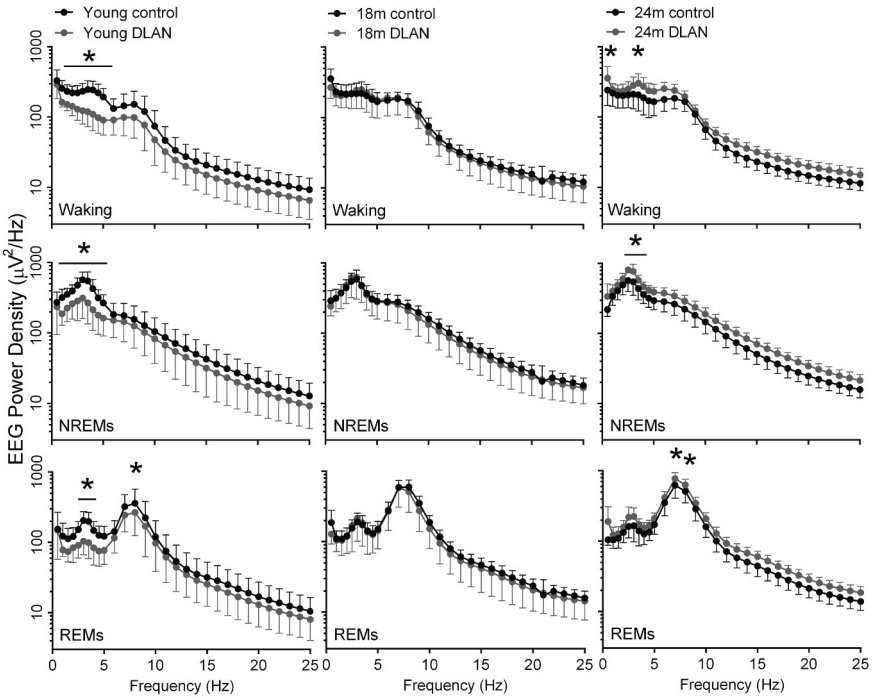

Figure 8.7: EEG power density in Waking, NREM and REM sleep for young, 18 and 24 months old mice in either control light:dark (black circles, n=11, 8, 9 respectively) or following three months exposure to light:dim-light-at-night (DLAN) conditions (gray circles, n=9, 8, 6 respectively) in the frequencies between 0.5 and 25 Hz computed for pooled values of the 24-h baseline day. Between 0.25 and 5.0 Hz, values were calculated in 0.5-Hz bins and between 5.25 and 25.0 Hz in 1-Hz bins. Black asterisks indicate significant differences between the control and the DLAN mice of each age group (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'treatment', 'EEG frequency bin').

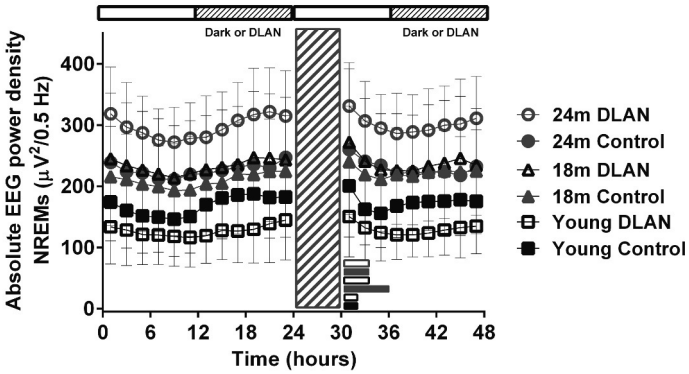

Figure 8.8: Time course of EEG slow-wave activity in NREM sleep (SWA, EEG power density between 0.5-4.0 Hz) for 24-h baseline, and 18-h recovery period following sleep deprivation (hatched bar) for young, 18 and 24 months old mice in either control light:dark conditions (n=11, 8, 9 respectively) or following 3 months exposure to dim-light-at-night (DLAN) conditions (n=9, 8, 6 respectively). The bars above each graph indicate the light-dark/dim light cycle. EEG SWA increases as a function of age and following long-term DLAN exposure it increases further in aged mice, while it decreases in young mice. Bars at the bottom (in line with the group legends colors) of the plot indicate differences between baseline day and recovery day for each group (post-hoc paired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVAs, main effects 'treatment', 'time of day', 'day').

Discussion

In the current study, we show that three continuous months of DLAN exposure has detrimental effects on sleep architecture, the sleep EEG, and rest-activity behavior across a large age spectrum. Regarding the sleep architecture, a characteristic delay of the 24-h vigilance state rhythm was found in all DLAN mice as compared to age-matched controls. An aged sleep phenotype was found in young mice exposed to long-term DLAN, and additional age alterations were apparent in aged DLAN mice. Interestingly, the factors DLAN, aging as well as their combination led to a decrement in recovery of the amount of lost NREM sleep following sleep deprivation. In contrast to the effect of DLAN on recovery of NREM sleep in young mice, REM sleep was almost fully regained in young mice exposed to DLAN. Notably, SWA in NREM sleep was differentially altered in young and aged mice. It increased as a function of age, which was further accentuated following three months of DLAN exposure in the 24 months old mice but not in the 18 months old. However, the lowest SWA levels among all groups were found in young DLAN mice. The data demonstrate, for the first time, that long-term DLAN exposure induces an aged behavioral sleep phenotype in young mice, and accentuates age-associated sleep changes in aged mice.

Sleep and behavior

Aging phenotype and circadian disruption

Pronounced sleep alterations following a long-term DLAN exposure, were found in

young mice as compared to age-matched controls. More waking and less NREM sleep were found in the light period, while the opposite was apparent in the beginning of the dim light period along with more REM sleep. Additionally, investigating the vigilance states across the 48-h among the three age groups, a similar characteristic pattern was evident in the young, 18 and 24 month old DLAN mice, showing a similar decrease in the daily amplitude of vigilance state rhythms following chronic DLAN exposure irrespective of age (Figure 5). A recent study in Wistar rats found a gradual reduction in rhythm amplitude in vigilance state distribution, starting from day 1 to day 14 of DLAN exposure [18]. Aged mice, as shown earlier, are characterized by sleep changes such as more NREM sleep and less waking during the dark/active period as well as less REM sleep at the end of the light/inactive period [10, 11, 12, 13, 14, 9, 8]. Our data demonstrate that, following chronic exposure to DLAN, behavioral sleep features converge to an aged sleep phenotype.

Behavioral data in our study demonstrate that in the course of aging the amount of locomotor activity decreases. Moreover, with the introduction and chronic exposure to DLAN, a delay in the phase angle of entrainment is noted in the 24-h rest-activity rhythm across all ages, which is also evident in the delay of each vigilance state in the 2-h sleep-wake values (Fig.1 and Fig. 2). Delayed temperature rhythms were also found after only 2 weeks of DLAN exposure in the grey mouse lemur [19]. In addition to delayed rhythms, the rhythm strength is also found attenuated following DLAN exposure in all groups, suggesting that DLAN affects the circadian clock and particularly the magnitude of the 24-h component [18, 20]. Notably, DLAN adds more effects in the aged circadian clock, decreasing further the strength of the circadian rhythm.

Although mice and humans obtain different characteristics with reponse to light, with the former being nocturnal and the latter being diurnal, our study has translational value regarding sleep and circadian disturbances owing to DLAN. DLAN exposure at the dark period in mice can affect the circadian clock and induce sleep alterations that impact not only the active, but, as a consequence the inactive period too. Generally, the rodent and human circadian timing system share many common features. In particular, the SCN rhythm of firing activity and neuropeptide expression is largely similar between nocturnal and diurnal mammalian species [24]. The present study suggests long-term DLAN in humans may influence their circadian clock in a similar way to mice, showing rhythm disturbances and affecting consequently sleep architecture. Future studies in diurnal animals may validate our hypotheses.

We found in our study that the NREM sleep lost during sleep deprivation was not fully regained in either of the experimental groups. In general, if a sleep homeostatic response occurs, a complete recovery of the amount of sleep is not necessary as sleep will be intensified, with high SWA during recovery [25]. However, young mice, chronically exposed to DLAN, showed an attenuated response to sleep deprivation compared to age matched controls and recovery sleep in this group resembled that of the aged mice exposed to DLAN. Aging can modify the response to sleep deprivation on NREM sleep amount and the pattern of recovery sleep duration [11]. As we show in our results, the homeostatic response declines in aging, and when alterations in light, such as DLAN, are added, this response is further reduced. In contrast to NREM sleep lost, REM sleep lost during the sleep deprivation was fully recovered in the young mice, even following chronic DLAN exposure. This is in contrast to the aged mice, as also reported earlier [11]. There-

fore, the mechanism of REM sleep homeostatic regulation may differ from NREM sleep regulation, as the former is not affected by DLAN exposure in young mice.

The sleep EEG: Accentuated age-induced characteristics

In addition to alterations found in young mice in slow-wave and theta frequencies in the waking, NREM and REM sleep spectra, which could reflect a general degradation in connectivity in EEG generating brain areas, as for instance the cortex and the hippocampus, it is evident in the present study that long-term DLAN exposure affects the sleep EEG in the course of aging. Compared to control, 18 months old mice did not show any changes in the EEG spectra when exposed to DLAN, but 24 months old mice demonstrated an increase in activity in the slow frequencies of waking and NREM sleep spectra as well as in theta frequencies in the REM sleep spectrum. This is in contrast with the findings in young mice, which are characterized by a general decrease in power following chronic DLAN exposure. Aged DLAN mice likely show increased drowsiness as indicated by the increase in activity in the slow-waves in the waking spectra [26, 27]. In a parallel way, a further increase in slow-waves in NREM sleep suggests that aging and DLAN exposure act synergistically. In particular, it is known that aged mice show an increased power in the slow-wave range of the NREM sleep EEG [8, 9]. Taking into consideration that following moderate physical activity, being considered a healthy intervention that can ameliorate several body and brain characteristics, EEG SWA decreases in aged mice [5], while it increases following long-term high-caloric diet in aged mice [28], it can be suggested that DLAN accentuates aging characteristics, as EEG SWA increases in 24 month old DLAN mice.

Concerning the latter assumptions, the question arises whether the SWA decrease found in the young mice implies a beneficial or detrimental effect of DLAN on the sleep EEG (Figure 8). Although it has been demonstrated that following the healthy intervention of running wheel, EEG SWA is decreased in an analogous way in both young and aged mice [5], here, the decrease in EEG SWA in young mice following long-term DLAN exposure, stems from the general attenuation in EEG power in all the prominent frequencies, including theta activity during waking and REM sleep, and therefore it is probably not an indication of improved health, but probably of a decrease in brain network integrity. We have suggested previously that aged mice have altered brain network properties compared to young animals, with overall reduced plasticity and possibly increased network connectivity in local circuits and weaker synchronization at the global level [9], resulting in higher SWA during NREM sleep. Additionally, all the EEG SWA responses to environmental changes, such as a high caloric diet or increased physical activity [5, 28], is less robust in aged compared to young animals. Accordingly, following chronic DLAN exposure, the cortical network at the local level is possibly only modestly affected in the aged mice, showing simply an increase in brain aging phenotype with more SWA in the EEG seen in the 24 months old mice, since generally the local cortical neural dynamics are not impaired during healthy senescence [8]. In contrast, the brain network of young mice, highly plastic and globally synchronized, is more profoundly affected at many levels, with a complicated response as an output that indicates overall an enhanced susceptibility to external inputs.

In conclusion, our data indicate that deleterious effects on sleep prevail following long-

term DLAN exposure across all ages. A diminished amplitude of the 24-h vigilance state rhythm is apparent in young mice exposed to DLAN, similar to aged mice. Additionally, disrupted circadian rhythms are found with a characteristic delay of activity in all age groups. Notably, aging in combination with long-term DLAN exposure augmented the age-associated sleep characteristics. Particularly with regards to the sleep EEG, EEG SWA was found to be further increased in 24 months old mice, and the response to sleep deprivation was attenuated in both 18 and 24 months old mice. A different effect on sleep EEG was evident in young mice compared to the old; this consisted of a general EEG power attenuation in the prominent frequencies in all vigilance states as well as in the decreased EEG SWA pattern compared to all groups. In accordance with earlier studies investigating the effects of DLAN on general health, we demonstrate that chronic DLAN exposure disturbs the circadian clock and alters sleep and EEG characteristics across a large age span.

Competing interests

Authors declare no conflicts of interest.

Author contributions

T.DB. designed research; M.P. and T.DB. performed research; M.P. and T.DB. analyzed data; and M.P. and T.DB. wrote the paper.

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Table 8.1: Statistical analysis: two or three-way analysis of variance (ANOVA) was conducted to test the effect of age, treatment and sleep deprivation (see text for more details). Asterisks following reported p values indicate significance (* p<0.05).

Table A1. Detailed statistics			
Figure	Vigilance state	Two- or three-way ANOVA Interaction factors	Main factors
2	Waking (6 months old mice)	'treatment*day*time of day' F(11,432)=1.5 p=0.13 with 'treatment*time of day' p<0.0001*	'treatment' p=0.255 'day' p<0.0001* 'time of day' p<0.0001*
	NREM sleep (6 months old mice)	'treatment*day*time of day' F(11,432)=1.63 p=0.088 with 'treatment*time of day' p<0.0001*	'treatment' p=0.005 * 'day' p<0.0001* 'time of day' p<0.0001*
	REM sleep (6 months old mice)	'treatment*day*time of day' F(11,432)=0.69 p=0.747 with 'treatment*time of day' p<0.0001*	'treatment' p<0.0001* 'day' p=0.013 * 'time of day' p<0.0001*
2	Waking (18 months old mice)	'treatment*day*time of day' F(11,336)=0.954 p=0.489 with 'treatment*time of day' p<0.0001*	'treatment' p=0.219 'day' p<0.0001* 'time of day' p<0.0001*
	NREM sleep (18 months old mice)	'treatment*day*time of day' F(11,336)=0.859 p=0.581 with 'treatment*time of day' p=0.003 *	'treatment' p=0.099 'day' p<0.0001* 'time of day' p<0.0001*
	REM sleep (18 months old mice)	'treatment*day*time of day' F(11,336)=0.672 p=0.765 with 'treatment*time of day' p<0.0001*	'treatment' p=0.424 'day' p<0.0001* 'time of day' p<0.0001*
2	Waking (24 months old mice)	'treatment*day*time of day' F(11,306)=0.84 p=0.6 with 'treatment*time of day' p=0.011 *	'treatment' p=0.279 'day' p<0.0001* 'time of day' p<0.0001*
	NREM sleep (24 months old mice)	'treatment*day*time of day' F(11,306)=0.894 p=0.547 with 'treatment*time of day' p=0.047 *	'treatment' p=0.114 'day' p<0.0001* 'time of day' p<0.0001*

	REM sleep (24 months old mice)	'treatment*day*time of day' F(11,306)=0.642 p=0.792 with 'treatment*time of day' p=0.002 *	'treatment' p=0.4 'day' p<0.0001* 'time of day' p<0.0001*
3	Waking (6 months old mice)	'treatment*day*time of day' F (1,72) = 0.0001 p=0.986 with 'treatment*Light-Dark' p<0.0001 *	'treatment' p=0.141 'Light-Dark' p<0.0001 * 'day' p=0.012 *
	NREM sleep (6 months old mice)	'treatment*day*time of day' F (1,72) = 0.034 p=0.855 with 'treatment*Light-Dark' p=0.001 *	'treatment' p=0.013 * 'Light-Dark' p<0.0001 * 'day' p=0.087
	REM sleep (6 months old mice)	'treatment*day*time of day' F (1,72) = 0.353 p=0.554 with 'treatment*Light-Dark' p=0.013 *	'treatment' p=0.002 * 'Light-Dark' p<0.0001 * 'day' p<0.0001 *
3	Waking (18 months old mice)	'treatment*day*time of day' F (1,56) = 0.006 p=0.937 with 'treatment*Light-Dark' p=0.039 *	'treatment' p=0.586 'Light-Dark' p<0.0001 * 'day' p=0.361
	NREM sleep (18 months old mice)	'treatment*day*time of day' F (1,56) = 0.0001 p=0.999 with 'treatment*Light-Dark' p=0.086	'treatment' p=0.415 'Light-Dark' p<0.0001 * 'day' p=0.888
	REM sleep (18 months old mice)	'treatment*day*time of day' F (1,56) = 0.063 p=0.8	'treatment' p=0.482 'Light-Dark' p<0.0001 * 'day' p=0.016 *
3	Waking (24 months old mice)	'treatment*day*time of day' F (1,51) = 0.006 p=0.936	'treatment' p=0.315 'Light-Dark' p<0.0001 * 'day' p=0.003 *
	NREM sleep (24 months old mice)	'treatment*day*time of day' F (1,51) = 0.007 p=0.935	'treatment' p=0.182 'Light-Dark' p<0.0001 * 'day' p=0.002 *
	REM sleep (24 months old mice)	'treatment*day*time of day' F (1,51) = 0.001 p=0.971	'treatment' p=0.653 'Light-Dark' p<0.0001 * 'day' p=0.3
4	NREM sleep	'age*Light-Dark*day' F(2,88)=0.6 p=0.55 with 'age*Light-Dark' p=0.006	'age' p=0.339 'Light-Dark' p<0.0001 * 'day' p=0.024 *
	REM sleep	'age*Light-Dark*day' F(2,88)=0. 593 p=0.555	'age' p=0.634 'Light-Dark' p<0.0001 * 'day' p=0.005 *

5	REM sleep (DLAN only mice)	'age*time of day*day' F (22,480) = 0.366 p=0.997	'age' p<0.0001 * 'time of day' p<0.0001 * 'day' <0.0001 *
6	Cumulative REM sleep (all DLAN mice)	'age*time of day' F(46,480)=0.34 p>0.99	'age' p<0.0001 * 'time of day' p<0.0001 *
7	Waking (6 months old mice)	'treatment*EEG frequency bins' F(29,540)=4.9 p<0.0001 *	'treatment' p<0.0001 * 'EEG frequency bins' p<0.0001 *
	NREM sleep (6 months old mice)	'treatment*EEG frequency bins' F(29,540)=5.93 p<0.0001 *	'treatment' p<0.0001 * 'EEG frequency bins' p<0.0001 *
	REM sleep (6 months old mice)	'treatment*EEG frequency bins' F(29,540)=1.348 p=0.1082	'treatment' p<0.0001 * 'EEG frequency bins' p<0.0001 *
	Waking (24 months old mice)	'treatment*EEG frequency bins' F(29,390)=1.5 p=0.0557	'treatment' p<0.0001 * 'EEG frequency bins' p<0.0001 *
	NREM sleep (24 months old mice)	'treatment*EEG frequency bins' F(29,390)=2.2 p=0.0006 *	'treatment' p<0.0001 * 'EEG frequency bins' p<0.0001 *
	REM sleep (24 months old mice)	'treatment*EEG frequency bins' F(29,390)=1.15 p=0.2786	'treatment' p<0.0001 * 'EEG frequency bins' p<0.0001 *
8	Slow-wave activity (SWA) in NREM sleep (all groups)	'treatment*time of day*day' F (55,1033)=0.093 p>0.99	'treatment' p<0.0001 * 'time of day' p=0.057 'day' p=0.006 *
	Slow-wave activity (SWA) in NREM sleep (6 months old control mice)	'time of day*day' F(8,89)=11.05 p<0.0001 *	'day' p=0.05 'time of day' p=0.93
	Slow-wave activity (SWA) in NREM sleep (18 months old control mice)	F(8,63)=5.48 p<0.0001 *	'time of day' p=0.9995 'day' p<0.0001 *
	Slow-wave activity (SWA) in NREM sleep (24 months old control mice)	'time of day*day' F(8,66)=9.46 p<0.0001 *	'time of day' p=0.9988 'day' p<0.0001 *

8	Slow-wave activity (SWA) in NREM sleep (6 months old DLAN mice)	'time of day*day' F(8,71)=2.447 p=0.0212 *	'time of day' p=0.99 'day' p=0.31
	Slow-wave activity (SWA) in NREM sleep (18 months old DLAN mice)	'time of day*day' F(8,63)=11.59 p<0.0001 *	'time of day' p=0.99 'day' p=0.0026 *
	Slow-wave activity (SWA) in NREM sleep (24 months old DLAN mice)	'time of day*day' F(8,45)=8.388 p<0.0001 *	'time of day' p=0.99 'day' p=0.0576
	Slow-wave activity (SWA) in NREM sleep (6 months old DLAN mice)	'time of day*day' F(8,71)=2.447 p=0.0212 *	'time of day' p=0.99 'day' p=0.31
	Slow-wave activity (SWA) in NREM sleep (18 months old DLAN mice)	'time of day*day' F(8,63)=11.59 p<0.0001 *	'time of day' p=0.99 'day' p=0.0026 *
	Slow-wave activity (SWA) in NREM sleep (24 months old DLAN mice)	'time of day*day' F(8,45)=8.388 p<0.0001 *	'time of day' p=0.99 'day' p=0.0576

Chapter 9

Sleep and Aging: a Genetic Model

Sleep in a premature aging mouse model

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Abstract

The quality of circadian rhythms and sleep deteriorate in the course of aging, inducing alterations in the overall behavior and physiology of an organism. In the field of sleep research, aging comprises a strenuous, time-consuming and subsequently not profoundly studied area. In the present study, we investigated sleep and the sleep electroencephalogram (EEG) in the Xpg-/- premature aging mouse model (maximum life expectancy: 18 weeks). We conducted continuous 48-h sleep recordings in mice with or without the premature aging phenotype (knockout vs. wildtype, n=6 for each group) at seven and fifteen weeks of age, corresponding to young adult and old age of the Xpg-/- mice respectively. We observed less waking and more non-rapid-eye-movement (NREM) sleep in the first half of the dark period and attenuated REM sleep during the light period, sleep architectural characteristics analogous to naturally aged mice. However, absolute EEG power density in all vigilance states was found to be diminished in the Xpg-/- mice. The latter is not found in normally aged mice, suggesting that sleep quality and brain integrity may be compromised in the Xpg-/- mouse model beyond normal aging. Notably, during the recordings of the mutant mice, torpor-like behavior was observed in the aged group. This mutation is in accordance with an accelerated aging phenotype, as far as sleep architecture is concerned, however our data suggest a different regulation of these sleep characteristics since XPG deficiency is driving general physiological aging into a condition, not seen in the naturally aged mice.

Keywords: sleep architecture, mutation, aging, electroencephalogram, EEG power density

Introduction

Almost all species have developed an endogenous circadian clock with a period of approximately 24 hours, which in mammals, is located in the suprachiasmatic nucleus of the anterior hypothalamus [1]. In addition to regulation by the internal biological clock, sleep is regulated by a homeostatic process which is dependent on prior waking and sleep [2, 3]. Aging is associated with a deterioration of circadian rhythms in physiology and behavior which is accompanied by a decrease in circadian amplitude [4, 5]. In parallel, it has been demonstrated that aged mice, compared to young mice, sleep more during the dark period and show an increase in slow-wave-activity in non-rapid-eye-movement (NREM) sleep, a marker of the homeostatic sleep process [6, 7, 4, 8, 9, 10, 11].
One theory on aging supports the idea that active free radicals, normally produced in the organism, damage the cellular components, ultimately leading to cell death and tissue dysfunction resulting in an aging phenotype [12]. DNA is one target of these oxidants and it has recently been shown that DNA repair mechanisms are important factors to slow down the aging process [13]. Although naturally aged animals comprise the first choice in aging research, there is a need for successful shorter timespan studies, since naturally aged laboratory rats and mice live for approximately two years. Therefore, several animal models with defects have been developed that have a shorter life expectancy [14]. Such models can help to identify the molecular basis for specific aspects of age-related phenomena. A defect in one particular nucleotide excision repair protein can lead to a defect in transcription-coupled repair and/or global-genome repair pathways [14]. These models recapitulate the human xeroderma pigmentosum (XP) syndrome; in most cases, animals with complete deficiency of XP complementation Group G protein (XPG) have a very short life span and display segmental progeria without developing tumors [15, 16].
In the current study, we investigate sleep and the sleep electroencephalogram (EEG) in a Xpg-/- premature aging mouse model, in order to test its validity towards a potential future replacement for aging studies in sleep research as well as to test which aspect of age-related sleep disorders is determined by deficits in DNA repair.

Materials and methods

Animals

For the experiments, XPG knockout mice (Xpg-/-, n=6) and age-matched wild type littermates (Xpg+/+ , n=6) (Erasmus University Medical Center, Rotterdam, the Netherlands) were used. Xpg-/- mice were obtained by crossing Xpg+/- mice with a pure C57BL6J and pure FVB backgrounds to yield Xpg-/- pups with a F1 C57BL6J/FVB hybrid background. The generation, genotyping and characterization of Xpg-/- mice has been previously described in detail [16]. Mice were group housed under controlled conditions (12:12 h light:dark cycle; lights on at 10:00) with food and water ad libitum in a temperature controlled room (20-23 °C). At the age of approximately six weeks, when surgeries were conducted, mice were individually housed.
All animal experiments were approved by the Animal Experiments Ethical Committee of the Leiden University Medical Centre and Erasmus Medical Center of Rotterdam (the Netherlands) and were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

Surgeries

Under deep anesthesia (Ketamine 100 mg/kg; Xylazine 10 mg/kg; Atropine 1 mg/kg), EEG recording screws (placed above the somatosensory cortex and cerebellum) and electromyogram (EMG) electrodes (placed on the neck muscle) (Plastics One, Roanoke, VA) were implanted as described previously [10, 17, 18]. The wire branches of all electrodes were set in a plastic pedestal and fixed to the skull with dental cement. The mice were allowed 10 days to recover.

EEG and EMG recordings

The EEG and EMG were recorded with a portable recording system (PS 1 system, Institute of Pharmacology and Toxicology, Zurich, Switzerland) as previously described [10, 17, 18]. Before each recording, a calibration signal (10 Hz sine wave 300 μV peak-to-peak) was recorded on the EEG and EMG channels. Both signals were amplified, conditioned by analogue filters and sampled at 512 Hz. The signals were filtered through a digital finite impulse response filter and stored with a resolution of 128 Hz. EEG power spectra were computed for consecutive 4-s epochs by a FFT routine within the frequency range of 0.25-25.0 Hz.
To record the EEG and EMG, animals were placed into experimental chambers and connected through a flexible cable and a counterbalanced swivel system to the recording setup, where conditions were similar to the home cage. Before starting the experiment, animals were allowed to adjust to the experimental conditions for a week. Subsequently, a 24-h baseline day was recorded, starting at lights on. Additionally, at the start of the second day, six hours of sleep deprivation were conducted by gentle handling, which is a mild intervention in order to induce elevated sleep pressure conditions [10, 17, 18, 19]. EEG and EMG were recorded continuously during sleep deprivation and, subsequently, for 18 hours to investigate sleep characteristics after sleep deprivation. Recordings were performed in all mice at the age of seven weeks (considering at this phase the mice as 'young') and they were repeated when mice reached the age of fifteen weeks (considering at this phase the mice as 'aged'). For comparison to natural aging, 6 and 24 months old naturally aged C57BL/6J mice (n=11 and n=9 respectively) were also used in identical experimental and housing conditions (*data previously obtained from* [17]). In the current study, the results of the baseline recordings are being reported and discussed.

Data analysis and statistics

Three vigilance states (Waking, NREM sleep and REM sleep) were scored offline in 4s epochs by visual inspection of the EEG and EMG signals as well as EEG power density in the slow-wave range, as described previously [19, 18, 10, 20]. For each epoch, the EEG power density in the delta (0.75-4.0 Hz) and theta band (6.25-9.0 Hz) and the integrated EMG value were graphically displayed on a PC monitor to enable scoring of the different vigilance states. The vigilance states were expressed as a percentage of artifact-free recording time. Epochs with artifacts were excluded from the subsequent data analysis of the power spectra, but vigilance states could always be determined. Number of episodes were computed in each vigilance state that were partitioned into ten exponentially increased duration bins from 4s to >1024s [10, 20, 21, 22, 23]. Two-way ANOVA was

performed to investigate potential alterations on vigilance states, vigilance state episode frequency, EEG power density with main factors 'group' (mutant/age), 'Light-Dark', 'time of day', 'episode frequency bin', 'EEG frequency bin', followed by paired and unpaired post-hoc Bonferroni-corrected student's t-tests.

Results

Sleep architecture

Young and aged Xpg-/- mice showed less REM sleep during both the light and dark periods, as compared to age-matched wild type mice, and particularly during the light period, aged Xpg-/- mice showed the lowest REM sleep levels compared to both the young mutant group and the wild type mice (Fig. 1, REM sleep: Two-way ANOVA, with factors 'group' and 'Light-Dark' $p<0.0001$ and interaction $p=0.0072$ and One-way ANOVA for 24-h BL: factor 'group' $p<0.0001$). Although the distribution of waking and NREM sleep was altered across the day, 12-h light and 12-h dark values, as well as 24-h values did not differ among the groups.

Across the 24-h recordings, 2-h values showed differences among the groups, with the old Xpg-/- mice being less asleep and more awake at the end of the light period, and more asleep and less awake at dark onset compared to both the young Xpg-/- mice as well as their age-matched wild type mice (Fig. 2) (Two-way ANOVA, interaction factors 'group*time of day', Waking: $p<0.0001$ with factors 'group' $p=0.0461$ and 'time of day' $p<0.0001$, NREM sleep: $p<0.0001$ with factors 'group' $p=0.2604$ and 'time of day' $p<0.0001$). Both the young and old Xpg-/- mice had significantly less REM sleep throughout the whole light period compared to the wild type mice, with the aged Xpg-/- mice showing the lowest REM sleep levels (Two-way ANOVA, interaction factors 'group*time of day', REM sleep: $p<0.0001$ with factors 'group' and 'time of day' $p<0.0001$).

In order to investigate whether the Xpg-/- premature aging mouse model demonstrates similar characteristics to naturally aged mice, we compared the data in the current study to sleep architectural data obtained previously from naturally aged C57BL/6J mice (6 and 24 months old) [17] (Fig. 3). Aged Xpg-/- mice shared similar sleep characteristics to 24 months old C57BL/6J mice during the dark period in waking, NREM and REM sleep. In the light period, however, differences emerged between the mutated aged and the naturally aged mice (Two-way ANOVA, interaction factors 'group*time of day', Waking: $p=0.1047$ with factors 'group' and 'time of day' $p<0.0001$, NREM sleep: $p=0.1979$ with factors 'group' $p=0.0001$ and 'time of day' $p<0.0001$, REM sleep: $p<0.0001$ with factors 'group' and 'time of day' $p<0.0001$). Particularly, at the end of the light period the aged Xpg-/- mice were more awake and they showed overall a lower amount of REM sleep during the 12-h light period compared to naturally aged 24 months old mice. Interestingly, the sleep patterns of the young Xpg-/- mice were very similar to the 6 months old C57BL/6J mice (Fig. 3, right panels). A small difference was only noted at the end of the light period, in which Xpg-/- mice were more awake (Two-way ANOVA, interaction factors 'group*time of day', Waking: $p=0.0006$ with factors 'group' $p=0.0248$ and 'time of day' $p<0.0001$).

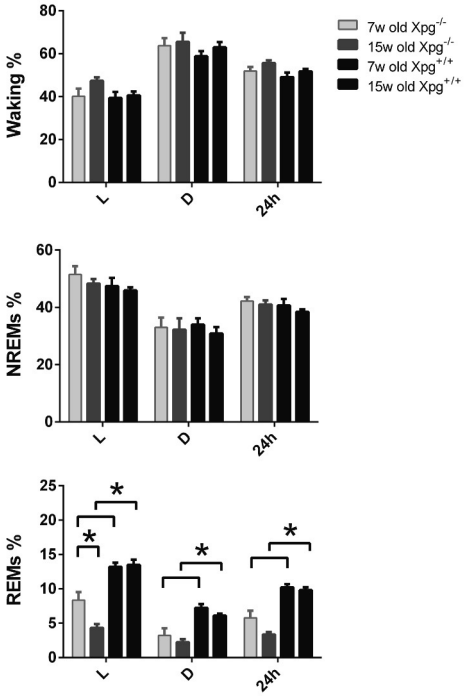

Figure 9.1: 12-h Light-Dark and 24-h vigilance state distribution (Waking, NREM sleep, REM sleep) of young and aged Xpg-/- mice (n=6) and age-matched wild type mice (Xpg+/+, n=6). Recordings were performed at the age of 7 weeks (young) and were repeated at 15 weeks (aged) for both knockout and wild type mice. Asterisks indicate significant differences between groups (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA main factors 'group' and 'Light-Dark').

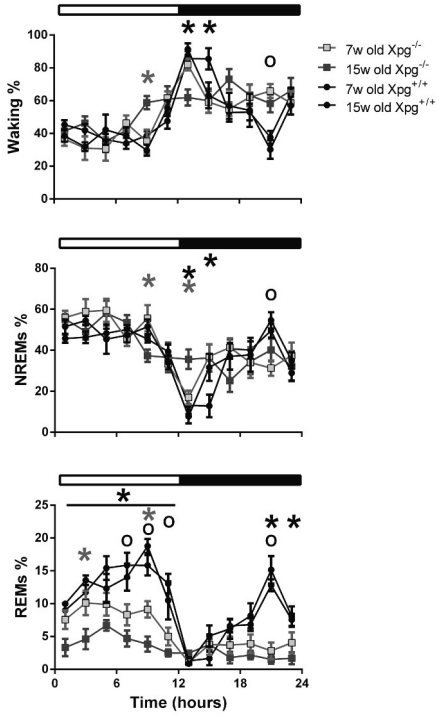

Figure 9.2: Time course of vigilance states (Waking, NREM sleep, REM sleep) for continuous 24-h recordings of young and aged Xpg-/- mice (n=6) and age-matched wild type mice (n=6). Lines connect 2-h mean values (± SEM) of waking, NREM and REM sleep. The black and white bars above each graph indicate the light-dark cycle. Gray asterisks indicate significant differences between young and aged Xpg-/- mice, black asterisks indicate significant differences between aged Xpg-/- mice and their age-matched wild type mice and circles significant differences between young Xpg-/- mice and their age-matched wild type mice (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA main factors 'group' and 'time of day').

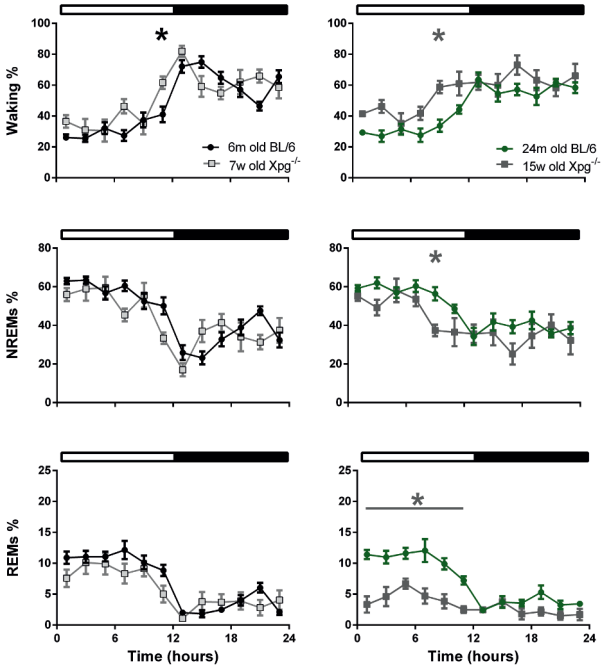

Figure 9.3: Time course of vigilance states (Waking, NREM sleep, REM sleep) for continuous 24-h recordings of young and aged Xpg-/- mice (n=6) and young control (6 months old, n=11) and naturally aged C57BL/6J mice (24 months old, n=9). Lines connect 2-h mean values (± SEM) of waking, NREM and REM sleep. The black and white bars above each graph indicate the light-dark cycle. Black asterisks indicate significant differences between mutant and naturally aged mice (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA main factors 'group' and 'time of day').

We additionally computed the number of episodes in each vigilance state that were partitioned into ten exponentially increasing duration bins from 4s to >1024s similar to [10, 20, 21, 22, 23] (Fig. 4). During both light and dark periods, Xpg-/- mice showed more short waking episodes (of 4s duration in the light and 4-16s duration in the dark) compared to wild type age-matched Xpg+/+ mice (Two-way ANOVA, interaction factors 'group*episode frequency bin', Light: $p=0.314$ with factors 'group' $p=0.0062$ and 'episode frequency bin' $p<0.0001$, Dark: $p=0.191$ with factors 'group' and 'episode frequency bin' $p<0.0001$). Notably, less REM sleep episodes were found in both young and aged Xpg-/- mice compared to age-matched wild type Xpg+/+ mice in almost all duration bins (8-128s duration during the light period and 8-32s duration during the dark period) (Two-way ANOVA, interaction factors 'group*episode frequency bin', Light: $p<0.0001$ with factors 'group' and 'episode frequency bin' $p<0.0001$, Dark: $p<0.0001$ with factors 'group' and 'episode frequency bin' $p<0.0001$).

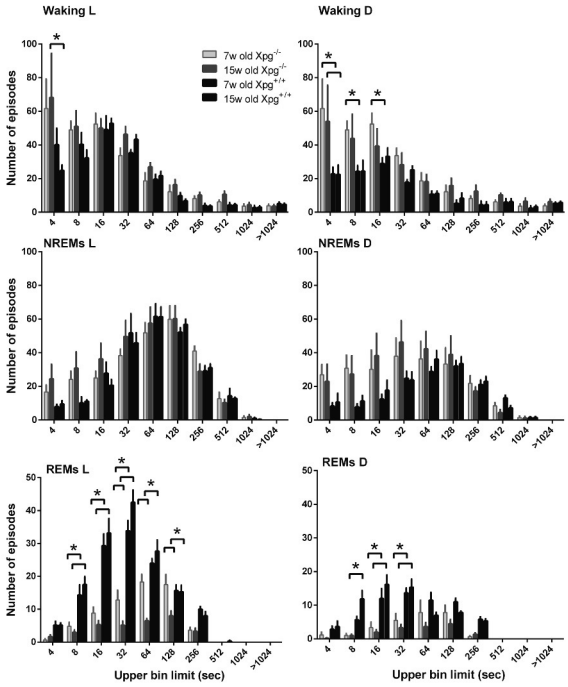

Figure 9.4: Histogram of vigilance state episodes frequency during the light (L) and dark (D) period. Episodes were partitioned in ten exponentially increased duration bins (upper bin limit from 4 to >1024s) for young and aged Xpg-/- mice (n=6) and their aged-matched wild type mice (n=6) in light and dark periods. Asterisks indicate significant differences between the groups (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'group', 'Light-Dark', 'episode frequency bin').

EEG power density between 0.5-25 Hz and slow-wave activity in NREM sleep

In order to evaluate whether similarities to normal aging could also be found in the EEG, we computed the absolute EEG power spectra in all vigilance states. Both young and aged Xpg-/- mice showed diminished power density levels in all vigilance states compared to Xpg+/+ mice, with aged Xpg-/- mice demonstrating the lowest overall levels (Fig. 5) (Two-way ANOVA, interaction factors 'group*EEG frequency bins', Waking: $p<0.0001$, NREM sleep: $p<0.0001$ and REM sleep: $p<0.0001$ with factors 'group' and 'EEG frequency bins' $p<0.0001$ everywhere). Additionally, the theta peak frequency in waking and REM sleep was significantly different between young mutant and age-matched wild type mice (Waking: 6.67 ± 0.21 Hz vs. 8.833 ± 0.167 Hz, REM sleep: 7 ± 0 Hz vs. 8 ± 0 Hz, $p<0.0001$) and between aged mutant and age-matched wild type mice (Waking: 5.833 ± 0.167 Hz vs. 9 ± 0 Hz, REM sleep: 6.333 ± 0.21 Hz vs. 8 ± 0 Hz, $p<0.0001$). The effects regarding the power spectra show that the quality of the EEG in the Xpg-/- premature aging mouse model is considerably attenuated.

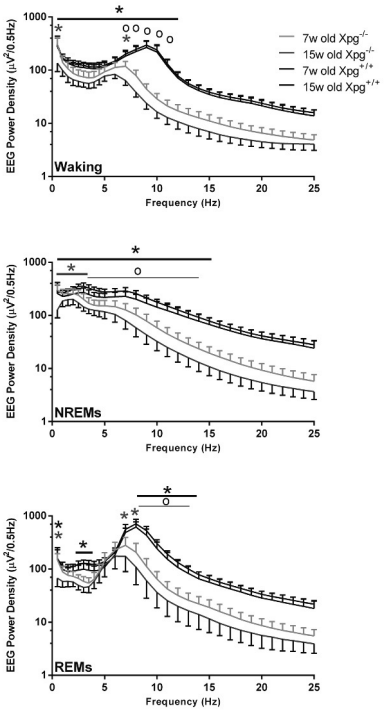

Figure 9.5: Electroencephalographic (EEG) power density in Waking, NREM and REM sleep for young and aged Xpg-/- mice (n=6) and their aged-matched wild type mice (n=6) (log $\mu V^2/Hz$, 0.5-25 Hz). Gray asterisks indicate significant differences between young and aged Xpg-/- mice, black asterisks indicate significant differences between aged Xpg-/- mice and their age-matched wild type mice and circles significant differences between young Xpg-/- mice and their age-matched wild type mice (post-hoc unpaired t-tests with Bonferroni multiple comparisons correction, $p<0.05$ after significant ANOVA, main effects 'group', 'EEG frequency bin').

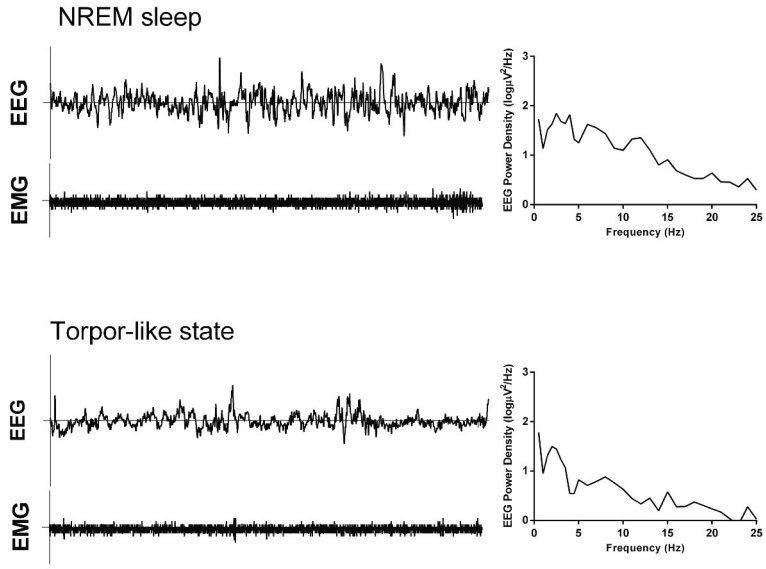

Figure 9.6: Representative example of raw electroencephalogram (EEG) and electromyogram (EMG) data of an aged Xpg-/- mouse during 16s of non-rapid-eye movement (NREM) sleep and a torpor-like state (left panels) with the corresponding EEG power density spectra (right panels).

Torpor-like state

In the course of the experiments, we encountered a phenomenon in the aged Xpg-/- mice. The recorded data suggested the occurrence of a torpor-like particularly at the end of the dark period, in which hypothermia was evident. The animals were found with low skin temperature (personal observations), and EEG amplitude was markedly reduced (representative examples in Fig. 6 and Fig. 7). Similar characteristics were earlier found in the Djungarian hamster, in which EEG was recorded during and after daily torpor (representative example of a Djungarian hamster in [24], Fig. 8).

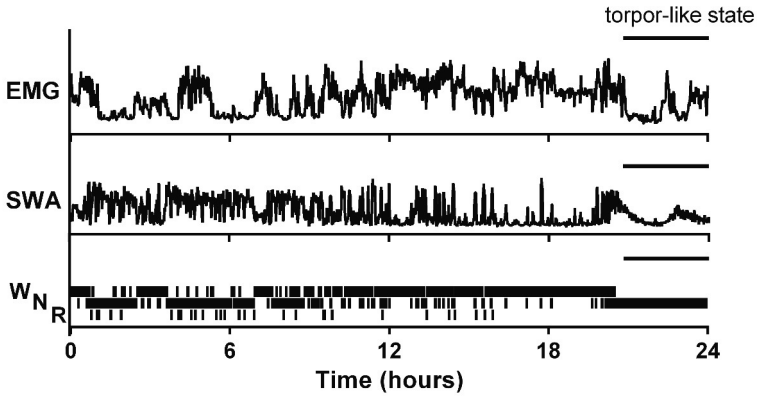

Figure 9.7: Representative example of an aged Xpg-/- mouse that developed a torpor-like state during the electroencephalogram (EEG) recordings. 1-min average during 24-h recordings of the electromyogram (EMG) with the corresponding slow-wave activity in NREM sleep (SWA, EEG power between 0.5-4.0 Hz) and hypnogram denoting the vigilance state (Waking, NREM sleep, REM sleep). At the end of the dark period, a torpor-like state was noted.

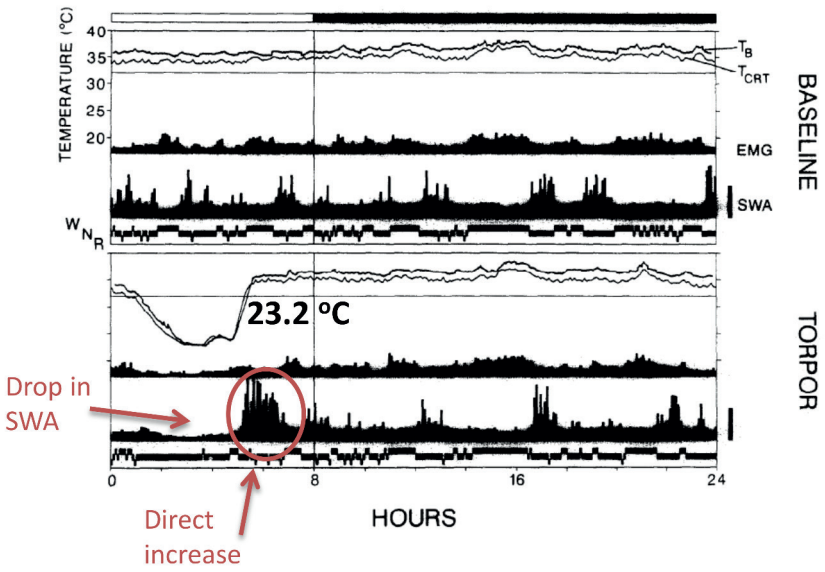

Figure 9.8: Representative continuous 48-h recordings of body temperature (T_B), cortical temperature ($T_C RT$), electromyogram (EMG), slow-wave activity (SWA) and vigilance states (W:waking, N:NREM sleep, R:REM sleep) of a Djungarian hamster (8:16 Light:Dark) during and after daily torpor (Adapted from [24]).

Discussion

The complexity of the aging process necessitates a concrete and solid animal model. The aim of the present study was to characterize the sleep parameters of a novel mutant mouse model whose DNA repair deficiency might render it suitable for aging research. We conclude that DNA repair deficiency leads to shared common features with naturally aged mice, regarding the architecture of sleep, consisting mainly of increased NREM sleep in the dark period and a decrease in REM sleep, but not to EEG features. We have thereby identified a mechanistic target likely able to alter sleep, simulating aging characteristics. Nevertheless, an exacerbated aging condition is possibly developed not apparent in naturally aged mice.

Many progeroid characteristics have been clearly reported regarding the Xpg-/- mice, however, a complete picture is still lacking [16]. At late embryonic stage, as earlier elaborately described, Xpg-/- mice have the same size and weight as wild type and heterozygote littermates [16]. In contrast, after birth, they show reduced growth and weight gain compared to controls, and stop growing at 6-8 weeks when their body weight is about 70% of that of wild type littermates. From 10-11 weeks, body weights declines and the Xpg-/- mice become progressively cachectic, where at 14 weeks all Xpg-/- mice are severely runted, with natural death in the following weeks (up to 18 weeks) [16]. In the current study, in order to explore sleep in the Xpg-/- mouse model, we performed EEG and EMG recordings in mice at the age of 7 and 15 weeks. Since these mice stabilize their full growth at the age of 6-8 weeks, recordings at that age were considered comparable to young adult. To characterize sleep in mice with fully expressed aging features, we repeated the recordings at the age of approximately 15 weeks. Although the background of Xpg-/- mice is two-fold, having crossed Xpg+/- mice with a pure C57BL/6J and pure FVB backgrounds, only data for young and aged C57BL/6J mice (as obtained in [17]) as well as young FVB mice [25] have been documented, whereas experiments regarding aged FVB mice are lacking. Therefore, in the present study, we compared the data from the Xpg-/- mice to naturally aged C57BL/6J mice. We found that aged Xpg-/- mice show sleep architectural characteristics similar to naturally aged mice that differed from both young Xpg-/- and their age-matched wild type littermates. They consisted of increased NREM sleep in the dark phase and decreased REM sleep during the light phase, similar to naturally aged mice [10, 11]. Albeit the similarities, the decreased amplitude of the 24-h vigilance state rhythm of the aged Xpg-/- mice led to increased wakefulness at the end of the light period and a lower overall amount of REM sleep during the 12-h light period compared to 24 months old C57BL/6J mice, likely exacerbating an aging phenotype. When compared to 6 months old C57BL/6J mice (Fig. 3), young Xpg-/- mice showed very similar sleep-wake patterns, validating their young nature as far as sleep architecture is concerned.

In contrast to sleep architecture, EEG power density spectra in waking, NREM sleep and REM sleep were found to be clearly attenuated in both Xpg-/- mice age groups. These EEG features in Xpg-/- mice distinctly deviate from those in naturally aged mice [10, 11]. In the Xpg-/- mice, there is an overall reduction in all prominent frequencies of the EEG. In contrast, in naturally aging mice the EEG changes differently, with increased EEG activity in the slow-wave range during NREM sleep, and decreased theta activity, particularly during waking [10, 11]. Since the EEG has been characterized as a window

on the brain, and alterations in the EEG may show attributes in the direction of neuronal network integrity [10, 17, 26], the EEG features of Xpg-/- mice likely reflect deterioration of cortical network integrity at the young age of seven weeks. Interestingly, it has been shown that the cerebellum of the Xpg-deficient mice demonstrates a large number of atrophic Purkinje cells, with smaller and shorter dendritic trees than their wild type littermates as early as 4 weeks of age [16]. In addition, the amount of apoptotic cells has shown a significant increase in both the cerebrum and the cerebellum at 4 as well as 14 weeks of age [16]. These findings may explain the overall lower activity in the EEG power density spectra, found in the young Xpg-/- mice. Therefore, regarding the EEG parameters, although a further decrease in the power was noted in aged Xpg-/- mice compared to young, our data show a phenotype in Xpg-/- mice distinctly different from what was found in naturally aging mice.
The lower power density and also the slower theta frequency could also be caused by a reduction in body temperature [27, 28]. Interestingly, during the recordings a torpor-like state was found in some of the aged Xpg-/- mice. The animals had a reduced body temperature, a reduced EEG power density, and it seemed that the EEG was slower, similar to the features observed in spontaneous torpor [24, 27]. Generally, mammals, naturally entering torpor, cope with harsh environmental conditions (i.e. food scarcity and/or cold exposure) by reducing their core body temperature and their metabolic energy requirements [29]. However, the exact mechanisms regarding torpor still remain largely unknown [30, 31]. A recent study could evoke a torpor-like state in a non-hibernating rat with pharmacological intervention [32]. Additionally, studies have shown that C57BL/6 mice can enter a torpor-like state after hours of fasting [33]. In relation to aging, the finding that in outbred mice (HaZ:ICR) torpor was observed during the last two weeks of their life [34] may be of special importance. The same study showed that body temperature between these torpor bouts was considerably lower than normal body temperature. If the latter also occurs in Xpg-/- mice, this may explain the slower theta peak frequency during REM sleep and waking, which is known to be temperature dependent [27]. The data suggest that Xpg-/- mice show metabolic changes that result in decreased body temperature and the occurrence of torpor bouts similar to mice which are at the very end of their natural live. The extremely small size of the Xpg-/- mice, in congruence with severe motor deficits and muscle weakness, as well as progressively developed neurological symptoms [16], likely contributed in the induction of the torpor-like state. Since torpor may be induced owing to survival issues or in case of adversities in nature, we can deduce that aged Xpg-/- mice experienced an extreme metabolic condition.

Concluding remarks

A deeper comprehension of the interaction between aging and the circadian clock, as well as the impact of aging on sleep structure and intensity could lead to a direct benefit for the aging population. The use of genetic models with an accelerated aging phenotype could shorten the scientific timespan so as new insights may emerge in the aging field. Here, we show that the Xpg knockout model shares many similar characteristics to naturally aged mice, especially regarding the sleep architecture. Although the DNA repair deficiency, in the currently investigated model, may reveal specific age-related characteristics, the

sleep phenotype is partially incomplete. It seems that an exacerbated aging condition is developed, particularly apparent in the sleep EEG and the torpor-like condition induced, usually not seen in naturally aged mice.

Acknowledgments

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Chapter 10

General Discussion

During our life span, a great amount of time is spent in sleep. Although not fully conceived why, sleep is unambiguously an important process for the brain and the whole organism. In aging, in congruence with general health deterioration, rendering the organism more vulnerable to disease, sleep is also altered. Several environmental factors, to whom we are daily exposed, affect the sleep behavior from adulthood to aging. In this thesis, an attempt has been made in order to elucidate the way sleep and subsequently general health can be enhanced in the course of aging.

10.1 Sleep characteristics in the course of aging

In accordance with numerous alterations, beginning with biochemical changes at the molecular level, that eventually expand to encompass the cellular, tissue, and organ system level, aging is associated with sleep and circadian changes in humans and animals. **Chapter 5** is the first of the series of chapters in the current thesis which follows a basic but detailed approach on sleep effects in the course of aging in the mouse model C57BL/6J. In **Chapter 5** it is demonstrated that older mice displayed different sleep characteristics regarding sleep architecture and the sleep electroencephalogram (EEG) as compared to young mice. In particular, the older mice showed more non-rapid eye movement (NREM) sleep and less waking especially during the dark period, emerging from an increase in the number of long NREM sleep episodes and a decrease in the number of long waking episodes respectively, as well as less REM sleep at the end of the light period. Although the sleep features of older mice depicted in **Chapter 5** are in line with earlier studies on the same and other mouse strains, the associated mechanisms underlying the changes remain uncharted [1, 2, 3, 4, 5, 6]. As shown in Figure 2 of the introduction of this thesis (**Chapter 1**), sleep and wakefulness are regulated by different brain circuits in which monoaminergic neurons are the protagonists. Since alterations in neurotransmitters such as adenosine, hypocretin (orexin), glutamate, GABA and others or their receptor balance are likely to induce changes in the sleep-wake circuit, they have been proposed to play an important role in sleep changes seen in aging [7, 8].
A significant result obtained in **Chapter 5** is that sleep in older mice is characterized by increased EEG slow-wave activity (SWA, EEG power in 0.5-4.0 Hz) in NREM sleep and altered slow-wave morphology compared to young mice. This suggests that older mice possibly live under higher sleep pressure conditions. In addition to that, the slower decay rate in SWA in the first three hours after sleep deprivation in the older mice suggests a decreased efficiency in dissipating sleep pressure with age. The morphological slow-wave findings in the old mice including the altered slow-wave slopes, the decreased sigma activity (activity between 9-13 Hz) during the down state, and the decrease in the number of multipeak waves, point towards altered brain network properties, suggesting increased neuronal coupling at the local level between cortical neurons, at the expense of the global connectivity. Although increased slow-waves in NREM sleep were also found in older mice in a more recent study, validating our data, it was demonstrated that the local cortical neural dynamics and local sleep homeostatic mechanisms were not impaired during healthy senescence in mice [6].

Fragmented behavioral rest-activity patterns, as well as reduced amplitude of electrical activity rhythms in the suprachiasmatic nucleus (SCN) characterize old mice, indicating that aging substantially affects the circadian clock [9, 1]. Interestingly, behavioral activity alterations in mice arise at the age of 12 months [1] (Figure 10.1 A). Sleep alterations in the course of aging seem to follow a similar path (Figure 10.1 B,C). Characteristically, in figure 10.1, sleep parameters are compared between 6, 12, 18 and 24 months old mice that were sedentary their whole life: 12 months old mice show circadian period and locomotor activity levels similar to older mice, intermediate vigilance state characteristics between young and older mice and EEG SWA in NREM sleep similar to older mice, which significantly differs from young mice (unpublished data). Therefore, we can conclude that an aging phenotype in sleep and circadian rhythms is apparent relatively early in a mouse life, considering that 12 months corresponds to earlier than middle age in the mouse life span.

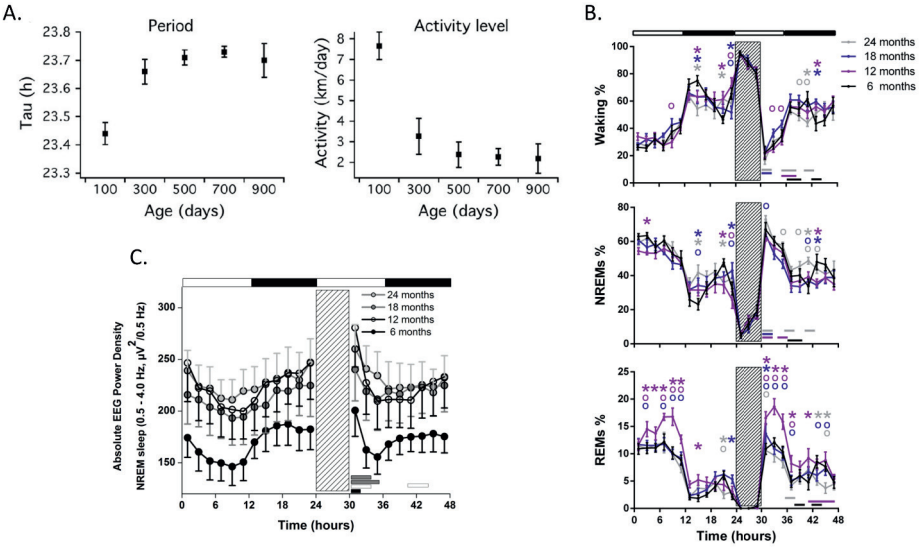

Figure 10.1: Sleep and behavioral alterations in the course of aging. A: Period and activity levels of mice at the age of 100, 300, 500, 700 and 900 days (Adapted from Farajnia et al., 2012 [1]), B: Time course of vigilance states for 48-h continuous sleep recordings in 6, 12, 18 and 24 months old mice without any environmental intervention, along with C: their EEG slow-wave activity in NREM sleep (see text for more details)

Interestingly, in contrast to the circadian clock changes, humans show different sleep patterns from mice as they grow older. In elderly humans, problems falling asleep, increased sleep fragmentation, decreased total sleep time and sleep efficiency, as well as attenuated slow-wave activity in NREM sleep (EEG SWA, power density between 0.5-4.0 Hz) are noted [10, 11, 12, 13]. The complete spectrum of age-related sleep alterations in aging was only recently fully documented, showing in most cases results in the opposite direc-

tion in laboratory mice as compared to humans. Nevertheless, these findings, including findings presented in **Chapter 5**, do not invalidate the mouse model as an important laboratory tool in sleep research. At the local cortical network level, there is a similarity between humans and laboratory animals since no evident alterations are apparent in the course of aging [6]. Markedly, disparities should be always taken into account when comparing between species, since considerable insights can be obtained. Animal models signify a necessity for the scientific world, however, it is shallow to consider that equal results will be obtained in humans in comparison to other animals. Through **Chapter 5**, therefore, it is established that in laboratory animals, in addition to attenuated behavioral activity, and altered amplitude and timing of circadian rhythms, sleep parameters are also affected as a function of age, albeit the mechanisms underlying these age-associated sleep changes remain elusive [2, 3, 1, 4, 5, 6].

The aging process, following the path from birth to the end of life, lasts for approximately two years in a mouse model, such as the C57BL/6J mouse strain. However, recent advances in technology and science warrant for successful shorter time-span studies. Thus, in addition to naturally aged mice, sleep parameters were investigated in a genetic mouse model of accelerated aging, portrayed in **Chapter 9**. In this chapter, we aimed at investigating a premature aging mouse model in order to assess its validity as a mouse model appropriate for sleep research. As detailed reported in **Chapter 1**, there are aging theories supporting the idea that active free radicals, normally produced in the organism, may induce aging by damaging the cellular components, ultimately leading to cell death and tissue dysfunction [14]. Since DNA comprises a major target for oxidants, DNA damage has to be restored through associated repair mechanisms. The latter are important mechanisms in the aging process, that, if overruled, could lead to premature aging and mortality [15]. An animal model based on the complete deficiency of DNA repair protein is the xeroderma pigmentosum Group G protein (XPG) knockout model [16]. Animals with complete deficiency of the XPG protein have a very short life span and progeria [17, 18]. Taking into account the significant properties of this mouse model for aging research, in **Chapter 9** we initially studied the sleep characteristics and subsequently compared them with data obtained in naturally aged mice.

Aged mutant mice shared similar sleep architectural characteristics to naturally aged mice, that differed from young mutants and age-matched wild type littermates (**Chapter 9**). These consist of less waking and more NREM sleep in the first half of the dark period and attenuated REM sleep during the light period. Despite these similarities with natural aging, the decreased amplitude of the 24-h vigilance state rhythm of the aged Xpg-/- mice led to more waking at the end of the light period and a lower overall REM sleep amount in the 12-h light period compared to 24 months old C57BL/6J mice, likely showing an exacerbated aging phenotype. In contrast to sleep architecture, EEG power density in all vigilance states across a wide frequency spectrum was found to be profoundly attenuated in young and aged Xpg mutant mice deviating from patterns seen in naturally aged mice. Since this was evident in all vigilance states, it can be suggested that sleep quality and brain integrity are likely compromised in the Xpg-/- mouse model even at the corresponding young adult age.

Notably, during the recordings, a torpor-like state was observed in the aged mutant mice, particularly at the end of the dark period, in which hypothermia was evident. The animals had low skin temperature and the EEG amplitude was markedly reduced. It is likely that

Xpg-/- mice show metabolic changes which may result in decreased body temperature and the subsequent occurrence of torpor bouts, in accordance with mice which are at the very end of their natural life [19, 20, 21, 22]. Concluding, although some similar sleep characteristics to naturally aged mice are apparent, XPG deficiency possibly alters physiological aging beyond what is usually found and therefore this premature aging mouse strain denotes a more extreme aging condition, not occurring under natural conditions.
Conversely to the first ideas in the neuroscience field developed some centuries ago, that the adult nervous system is rather hard-wired and non-resilient, the last decades it is established through laboratory animal and human studies, that the adult mammalian brain obtains plastic properties which can be continuously reformed by environmental input [23, 24]. In the two following sections we are discussing environmental inputs, commonplace in everyday life, that positively or negatively affect sleep and subsequently brain function during aging.

10.2 Factors that impair sleep and behavior

Aging is an unavoidable process that affects the development of organisms throughout the lifespan. Healthy aging comprises an utmost goal of every individual. However, an array of debilities emerges not only in pathological but also in healthy aging. As discussed earlier, sleep alterations are commonly viewed in aging. When the environment intervenes with aging, detrimental effects arise for general health that could consequently even accelerate or accentuate aging. **Chapters 2, 4, 7 and 8** aim at investigating sleep and circadian alterations following long-term high-caloric diet consumption and dim-light-at-night exposure.

10.2.1 High-caloric diet

Diet-induced obesity is a modern disease that affects big parts of the population worldwide. General health is compromised in people with obesity that can eventually lead to secondary diseases and ultimately to increased mortality even at a young age [25]. Obesity, in conjuction with physical inactivity, is likely to increase the risk for cardiovascular disease, Type 2 diabetes, hypertension, insulin resistance, neurodegeneration, dyslipidemia and certain cancers [26]. Additionally, obesity and metabolic disorder are accompanied by chronic low-grade inflammation, linked to alterations in brain function such as hypothalamic inflammation [27, 28, 29, 30]. One possible factor that may contribute to obesity is sleep behavior, owing to an increase in sleep debt due to modern lifestyle and working conditions [31, 32]. Concurrently, obesity directly or indirectly may induce sleep disturbances [33, 34]. Therefore, obesity and sleep are two interrelated entities that are investigated in **Chapters 2 and 7**.
In **Chapters 2 and 7** young adult (6 months old) and aged mice (18 months old) were fed exclusively with high-caloric diet (HCD) for three months, signs of obesity being evident even after the first week of this diet. An increase of almost 65% of the body weight was apparent following twelve weeks of treatment. In young HCD fed mice, moderate sleep architectural alterations were found compared to age-matched control mice, denoted by

an increased REM sleep amount emerging from an increased probability of consecutive NREM-REM sleep cycles. Instead of sleep fragmentation usually assumed in obese subjects, increased sleep consolidation was shown in the data, confirming earlier studies [35, 36, 37, 38]. Due to the increased REM sleep, and the close relationship between REM sleep and thermoregulation [39], we hypothesized that young mice fed chronically with high-caloric diet, would probably show an altered thermoregulatory activity along with modulated metabolic rate. Interestingly, recent findings on young HCD fed mice indicate that body temperature of obese mice is approximately 1 degree higher compared to lean mice, validating hence our hypothesis of a reduced thermoregulatory demand in the HCD fed mice [40].
It is known that aging leads to a redistribution of body fat with similar changes to the ones seen during HCD consumption [41, 42]. In addition, increased total fat mass and elevated levels of abdominal adipose tissue are noted at middle age, being characterized as risk factors for several diseases [41, 43]. Thus, the hypothesis was formed in **Chapter 7** that obesity and aging may act synergistically, negatively affecting sleep. The data demonstrated that sleep architecture was altered in 18-months old HCD treated mice. They showed increased NREM sleep and decreased waking, compared to age-matched controls, denoting a potentially enhanced aging phenotype in the sleep architecture.
In **Chapter 2** insights regarding the regulation of sleep were investigated, by applying parameter estimation analysis and mathematical modeling of the observed homeostatic sleep response. The observed homeostatic sleep response was visible in the EEG SWA, which is directly associated with the homeostatic sleep process. Young HCD fed mice in addition to an overall decrease in absolute EEG SWA during NREM sleep, showed a slower build-up of sleep pressure during waking and REM sleep. The data converge that HCD alters sleep homeostasis, rendering young mice less susceptible to prolonged waking. On the contrary, as it is shown in **Chapter 7**, older HCD fed mice showed increased levels of SWA in NREM sleep particularly in the slow component (0.5-2.5 Hz), as compared to both young HCD fed, young and aged controls. Although aging is the prevailing parameter, it seems that regarding SWA, obesity adds further effects in aged mice.
Besides the finding that functioning of the central clock remains intact following HCD [44], it seems that HCD, chronically consumed by young mice, provides short-term benefits by attenuating the burden of the sleep debt, since it reduces the effect of prolonged waking on subsequent EEG SWA in NREM sleep. Although, at first glance, the data seem to point against adverse effects of HCD on sleep and the circadian clock, particularly during young adulthood, the persistent increased weight, already associated with a multitude of diseases, along with aging is demonstrated to be remarkably detrimental for sleep, and general body and brain health in older animals.

10.2.2 Dim light at night

In modern society, in addition to dietary preferences and food availability, including ultra-processed and junk food that could eventually lead to obesity, there is a widespread use of artificial light which is also associated with various health disturbances. Although the invention of electrical light accounts for numerous advantages nowadays, light expo-

sure particularly at night has been linked with metabolic, immunological, and behavioral rhythm disturbances across a wide age spectrum [45]. In **Chapters 4 and 8**, we study the effects of dim light at night (DLAN) exposure on sleep, the sleep EEG and circadian behavior in young and aged mice.
The data showed that, following merely one night of DLAN exposure, there are altered sleep parameters, including a delay of vigilance state rhythms which increases as a function of DLAN exposure from one day to one month (**Chapter 4**). This is in accordance to earlier findings in rats [46] as well as temperature rhythm findings found to be accordingly delayed in lemurs [47]. After three continuous months of DLAN exposure, severe effects on sleep and rest-activity rhythms were evident in young mice, with the dynamics of the daily light-dark/dim amplitude being significantly distorted, similarly to aged mice exposed to DLAN (**Chapters 8**). Additional analysis on the rest-activity data of young mice revealed that chronic DLAN attenuates daily fluctuations corresponding to healthy physiology and negatively affects brain network's integrity [48].
Interestingly, the most pronounced changes in the sleep EEG were found in young mice, demonstrating a general power attenuation across all prominent frequencies (**Chapters 4 and 8**). In accordance with the diminished rhythmicity in rest-activity after three months of DLAN exposure, the spectral alterations suggested a reduced quality of brain cortical activity in all three vigilance states. The general decrease in EEG power density may be caused by a loss of thalamo-cortical synchronization due to prolonged DLAN exposure [49]. Nevertheless, the cerebral cortex is not just a passive receiver of synchronized delta potentials of thalamic origin, but these inputs are reorganized by the intrinsic properties and synaptic events in cortical circuits, therefore, alterations following DLAN exposure may also take place at the cortical level [49, 50]. After chronic DLAN exposure, EEG SWA in NREM sleep was differentially affected in 6, 18 and 24 months old mice, showing an attenuation in the young mice, and an increase in the 24 months old mice while no alteration was found in the 18 months old mice. Therefore, long-term DLAN exposure induces pernicious sleep and circadian effects in young and aged mice alike, impacting first the sleep regulatory system and brain integrity in young mice and second accentuating age-induced sleep characteristics in aged mice.
Aging is an inevitable process which can be accelerated or decelerated depending on our lifestyle choices. By opting to abstain or lessen exposure to agents detrimental for general health, the path towards longevity could be ensured. Because what determines normal longevity, is the temporal aspect and the actual quality of the process of aging. Diet and light constitute two considerable environmental factors in everyday life. **Chapters 2, 4, 7 and 8** discussed in this section are, therefore, dedicated to the investigation of the effects on behavior & sleep following unhealthy diet, such as HCD consumption, and light exposure, particularly DLAN. Through these chapters it is indicated that HCD and DLAN largely impact sleep and circadian behavior, the sleep EEG as well as the underlying network during adulthood as well as aging.

10.3 Factors that enhance sleep and behavior

It is widely known that, in the course of aging, several body and brain functions gradually decline irreversibly. Contrary to environmental factors that impair sleep and its function,

impeding eventually general health during aging, other factors induce antithetical effects. In **Chapter 6** of the present thesis, the effect of long-term physical activity is investigated on sleep, the sleep EEG and behavior, through a running-wheel available in the cages of young and aged mice.

10.3.1 Physical activity

Exercise is generally considered beneficial for health across all ages [51, 53, 54, 55]. During the last decades, a large body of literature has focused on benefits of aerobic exercise, on cognitive and executive function in children, whose brains are highly plastic [56, 58, 57]. Another sensitive age group that could value from external but not invasive tools are the elderly. Epidemiological studies have shown the advantageous relationship of physical exercise and cognition in pathological and normal aging, exerting beneficial effects on memory function as well as protecting from age-related cognitive decline [59, 60, 61, 62, 63]. An abstinence of sedentarism through a more active lifestyle that includes physical activity, has been demonstrated to improve cardiovascular health, enhance stress reduction, ameliorate or prevent depression and anxiety [51]. The mechanisms underlying the main effects of physical activity suggest neurogenesis, neurotrophic factors, angiogenesis and improvement of mood [52]. As far as sleep is concerned, exercise has been proposed as an alternative treatment in order to ameliorate potential sleep disturbances in both young and aged subjects [64, 65, 62, 66]. In order to assess whether sleep parameters in aging, elaborately depicted in **Chapter 5** can be counteracted, **Chapter 6** is dedicated to the long-term effects of exercise on sleep, since analogous studies in humans and laboratory animals are scarce.

Young and aged mice, used in this study, were provided with a running wheel in their cages, for voluntary use on a daily basis for one to three months. Since running wheels were permanently removed two weeks prior to the sleep recordings, the sustained effects of exercise were studied. Young mice provided with a wheel were more awake and slept less in the dark period compared to young controls. These effects resembled the results seen in mice that are concomitantly recorded with a running wheel [67, 68] showing that a sustained exercise effect exists even after the two weeks of wheel removal. In the course of aging, this effect was attenuated, mildly affecting the sleep architecture of 18 and 24 months old mice. Equivalently, biomarkers of brain activity and neurogenesis are altered owing to exercise in mice across all age groups studied, albeit only young animals are able to maintain a significant increase above baseline levels, after two or four weeks after the end of exposure to a running wheel [69, 70, 71].

The age-related increase apparent in the course of aging in EEG SWA levels, was found to be counteracted in **Chapter 6**. In particular, it was found that long-term physical activity diminished the age-associated EEG SWA towards levels similar to young sedentary mice (Figure 4 of **Chapter 6**). In contrast to sleep architecture, this effect of long-term voluntary exercise on EEG SWA was sustained for at least two weeks after removal of the running wheel across all ages. This shows that introduction of moderate activity even later in life induces significant changes towards a younger brain phenotype. Due to the revealing features of EEG SWA in all age groups, we conducted additional analysis using information-theoretic and machine-learning approaches and found that characteristic

information regarding age and exercise was enclosed in SWA. Furthermore, with cluster analysis, we could classify and accurately distinguish the different groups based solely on their SWA. Taking into account recent findings regarding local cortical neural dynamics as well as local sleep homeostatic mechanisms which probably remain unaffected in aging [6], our study suggests that global dynamics associated with age-induced alterations are mainly affected through regular physical activity leading to a potential 'younger phenotype' (**Chapter 6**).
In humans, shrinkage of medial temporal lobe structures has been found in middle-aged adults and older people [72, 73]. Hippocampal volume loss in particular, seen in aged humans, was found to be restored following one-year walking training [74]. Additionally, similar to our study, after short- and long-term exercise, SWA levels and slow-wave-sleep was altered in old humans, reaching levels closer to younger subjects [75, 76, 77, 64]. Concluding, age-matched, voluntary physical activity can lead to a 'younger phenotype', likely through delaying shrinkage, or even restoring the structure of various brain structures.
Aging inescapably occurs as a function of time in the organisms, in an asynchronous way in different brain areas, being characterized by a reduction in the reparative and regenerative potential in tissues and organs. The rate of the aging process is modulated by environmental factors and related to the neuronal-synaptic-molecular substrates of each area [8]. Henry David Thoreau writes about the time that flows *"Time is but the stream I go a-fishing in. I drink at it; but while I drink I see the sandy bottom and detect how shallow it is. Its thin current slides away, but eternity remains. I would drink deeper; fish in the sky, whose bottom is pebbly with stars."* Although we cannot overpass or overcome time and its outcome, life enrichment in the elderly, similar to environmental enrichment in animals, may comprise a key towards improvement of the quality of life sometimes lacking in really advanced age. Enrichment potentiates social interactions as well as learning, memory, sensory and motor stimulation, promoting plastic changes in the brain, known to be degraded in the course of aging [78, 79]. Our study in **Chapter 6** suggests that voluntary, long-term, age-matched exercise could attenuate the effects of sedentary behaviors and it could be prescribed as a first-order "medication" for general body as well as brain health augmentation throughout the whole age spectrum.

10.3.2 Caffeine

Caffeine is a xanthine alkaloid which is found in a variety of foods and beverages, such as chocolate, coffee, and tea. Coffee is the most commonly consumed drink after water worldwide [80]. Traditionally, it is believed that short- or long-term caffeine consumption disrupts sleep and may even induce health problems such as anxiety and increased stress, overwhelming fatigue, digestive disturbances, palpitations, high blood pressure and depression [81, 82, 83, 84]. Research has shown that caffeine ingested before sleep time may impact sleep by reducing the total sleep time and prolonging the sleep latency, with effects evident even with caffeine consumed in the morning [93]. Although a maximum daily intake of caffeine is recommended especially for children, adolescents and pregnant women, caffeine can have positive effects, increasing alertness and energy, well-being, relaxation, good mood and improved memory [91, 92, 83]. More particularly, caffeine has been used to treat apnea of prematurity, as a fatigue countermeasure by

truck drivers, shift workers, airline pilots and it is found in several medications against headache and appetite suppression [85, 86, 87, 88, 89, 90].

In **Chapter 3** we investigated the effects of acute and prolonged caffeine consumption on sleep and circadian parameters, by introducing caffeinated drinking water in the cages of the mice for up to two weeks. Validating the common belief that caffeine promotes arousal, the study confirms earlier findings that acute intake of caffeine increases waking with the concomitant diminished NREM sleep consolidation during the active phase [94, 95, 96, 97, 98, 99, 100, 101, 102, 103, 104, 105, 106]. While effects similar to the acute condition were found during the dark active period, following prolonged caffeine consumption, additional effects were found during the main sleep period with increased and deeper sleep. Interestingly, these effects were rather notable since caffeine has generally been considered to disturb sleep [107, 108]. A recent study supports adaptation following several days of caffeine intake in humans, showing that caffeine does not considerably shift melatonin and cortisol while it does not lead to an increased wake-promotion in the evening [109]. In accordance with spectral changes seen in mice provided with a running-wheel showing enhanced alertness, after chronic caffeine ingestion, the activity in the slow-wave range was markedly reduced while theta activity was accentuated [110, 111] suggesting that during waking, the animals were more active. Moreover, increased SWA was noted at the beginning of the light period, which together with the finding that NREM sleep duration was also increased during this period, suggests that sleep pressure is augmented. Sleep deprivation, additionally performed in this study, proved to be extremely challenging during chronic caffeine intake validating the notion of an increased sleep pressure in the first half of the light period.

It has been demonstrated that caffeine not only modulates sleep homeostatic mechanisms, but it also influences the circadian clock function [112, 113, 114]. Particularly, caffeine has been shown to increase circadian amplitude in vitro [115], while in addition it increases the influence of light on the SCN [114], which in turn may also lead to an increased circadian amplitude. Through **Chapter 3**, it is therefore suggested that an increase in the amplitude of the circadian clock, similar to the wheel access effect on the clock [116], likely underlies the increase in the 24-hour amplitude of the rest-activity and sleep-wake behavior, explaining this way the increase in SWA and sleep pressure at the beginning of the light period.

Chronic caffeine consumption has been linked with cognitive decline prevention, reduced risk of developing stroke, Parkinson's and Alzheimer's disease [117, 118, 119, 120, 121]. Since, in addition, caffeine amplifies cognitive performance, with an improvement in reaction time, vigilance and logical reasoning during extended periods with restricted opportunities for sleep [122], it can be suggested that daily caffeine intake can be part of a healthy balanced diet and its consumption does not need to be stopped in elderly people. Thus, in accordance with factors that enhance sleep and behavior such as the aforementioned effects of exercise, caffeine conversely to the traditional view may actually aid sensitive groups as for example the elderly by accentuating performance, alertness, increasing circadian amplitude and generally ameliorating sleep health. Future studies are needed in order to endorse this hypothesis for the elderly.

10.4 Biomarkers of brain age: inside EEG SWA in NREM sleep

Physiological aging is a complicated process whose molecular hallmarks and organ-specific physiological functions are affected by genetic, epigenetic, as well as environmental factors. In the current thesis, we focus on the environment and its effects on sleep and show that aging can be accentuated, accelerated or decelerated by the daily and long-term exposure to agents such as diet, physical activity and light, disentangling consequently the 'chronological' from the 'biological' age conceptions. Chronological age provides limited information regarding the complex processes that regulate aging. As a result, individuals with the same chronological age may vary in health, disease and disability, and therefore although coeval, they may differ in biological age [123, 124, 125]. Biological age does not perfectly correlate with time, since it is influenced by additional parameters as for example genetic background, disease, lifestyle. It has been demonstrated that even subjective perception of aging does not coincide with the chronological age and is associated with the process of brain aging and brain health, since feeling subjectively older likely reflects relatively faster aging brain structures [126, 127]. Therefore, the difference between chronological and biological or brain age may show advanced or delayed brain aging.

Biological information, which can be used to predict the brain age of an individual, has the potential to offer clinically relevant biomarkers, particularly useful for the elderly and the diseases associated with advanced age. The large neuroimaging datasets, in conjunction with newly developed machine learning techniques, have facilitated research regarding the estimation of brain age and biomarkers [128, 129, 130]. During the last decade, estimated brain age, primarily with the use of magnetic resonance image data, has been demonstrated to predict indicators of neurobiological aging, including cognitive impairment, obesity as well as diabetes [131, 132, 133, 135, 136]. Anatomical characteristics such as cortical thickness have also been used to successfully predict brain age, with a future application of an early prediction of neurocognitive disorders [137]. In addition to these features, brain EEG signals have been recently used in brain age prediction, since EEG traits, as for example EEG rhythms, alter as a function of age [138]. Age prediction from EEG has been studied with increased accuracy, using functional connectivity features from EEG resting-state recordings in order to correctly classify young and middle-age groups [139].

In addition to the aforementioned EEG attributes, sleep EEG can also provide elaborate information that could predict brain age, since significant changes are noted in it in the course of aging. Throughout the chapters of the present thesis, a common conjecture is the long-term effect of environmental factors on EEG SWA in NREM sleep. Depending on the positive or negative factor for general and brain health, effects on EEG SWA follow a specific direction in aged mice. In **Chapter 6**, we investigate the effect of long-term physical activity, a well-known advantageous agent that enhances general health, on sleep and the sleep EEG. We show that EEG SWA increases with age and attenuates with exercise, effects that are sustained even following some weeks after the cessation of the activity. Subsequently, taken into account these cross-age outcomes, we examine in this chapter whether EEG SWA contains information regarding the brain age of the mice. Thus, using machine learning techniques, we validate the hypothesis that characteristic

information concerning age and exercise is enclosed in SWA, enabling us to classify as well as successfully identify the different age groups based merely on their EEG SWA. Further studies in the present thesis (**Chapters 7 and 8**), exploring the deleterious effects of chronic high-caloric diet, including high-fat and high-sugar consumption, as well as continuous dim-light at night exposure on general and brain health, show that EEG SWA tends to follow the opposite direction compared to the results found following exercise. We propose, hence, that information derived from the sleep EEG, and particularly the EEG SWA, can be used as a biomarker of brain age in an analogous way to the studies depicted earlier. In accordance, recent studies confirm our considerations in relation to the biological information that can be obtained through the sleep EEG in general and the EEG SWA in particular which can be used to predict the brain age of an individual [140, 141].
EEG SWA is a marker of sleep need and sleep depth, and is profoundly affected by aging as it is shown in **Chapter 5** [142, 143, 6]. From a neuronal point of view, SWA reflects the dynamics of neuronal firing and more specifically, the ability of cortical neuron populations to engage in synchronized activity through synaptic connections [144]. Brain aging is generally influenced by progressive and regressive neuronal processes due to cell growth and death [145, 132]. In addition, environmental factors and health conditions of individuals, are likely to affect structural changes in the brain [146, 147, 148, 137]. Through biomarkers such as the EEG SWA, reflecting not only sleep-related changes but also functional brain changes, we could predict, identify, and eventually rectify the brain age so that general health is improved in aged as well as young individuals.

10.5 Concluding Remarks and Future Directions

The present thesis consists of studies dedicated in the investigation of environment and sleep in young adult and aged mice. The first part, including **Chapters 2, 3, 4**, delves into the long-term effects of high-caloric diet, both the acute and long-term effects of dim-light at night exposure as well as acute and prolonged caffeine intake on sleep and circadian parameters in young adult mice. The second part, consisting of **Chapters 5-9**, explores sleep and behavior in naturally and genetically modified aged mice in normal dietary, 12:12h light-dark, sedentary conditions as well as following prolonged high-caloric diet consumption, dim-light at night exposure and physical activity.
Throughout the results presented in the current thesis, the effects of environmental factors, aging as well as their combination on sleep, on circadian behavior and particularly EEG SWA provide contributions to sleep research. Effects are especially pronounced in young mice, showing that they are more susceptible to environmental changes, whereas, although present, effects on aged mice are overall more moderate. Long-term DLAN exposure in young mice is found to profoundly disrupt sleep and circadian behavior with characteristics similar to the ones found in aged mice (**Chapter 4**). Notably, a general EEG power attenuation in the prominent frequencies in all vigilance states likely reflects a widespread degradation in connectivity in EEG generating brain areas. In addition, chronic consumption of high-caloric diet led to an altered sleep regulation of young mice, showing short-term benefits in the case of sleep dept but being markedly detrimental on the long-term (**Chapter 2**). Following prolonged caffeine consumption and physical ac-

tivity, sleep and circadian behavior are positively substantially and similarly affected, with effects of exercise lasting for at least two weeks after activity cessation (**Chapters 3 and 6**). Although sleep and circadian behavior is mildly affected in aged mice as portrayed in the second part of the present thesis, the effects found particularly on the sleep EEG and the SWA suggest that aging in combination with chronic negative factors as dim-light at night and high-caloric diet act synergistically, accentuating age-related sleep effects with an impact on general and brain health, while physical activity in aged mice, comprising a positive lifestyle intervention, points towards a younger sleep phenotype promoting as a result body and brain health (**Chapters 6, 7 and 8**).

Nowadays, brain age prediction is an active research area, where brain age can be used in order to identify potential protective or deleterious factors for brain health as people age. Novel evidence suggests that an 'older'-appearing brain is linked to advanced physiological but also cognitive aging increasing concomitantly the risk of mortality [129]. According to the results of the present thesis, EEG SWA can be considered as a biomarker of brain age, marking distinct aging signatures that reflect beneficial or detrimental effects on body and brain health, due to specific lifestyle choices. Although owing to the underlying multisystem nature of the aging process, no single biomarker is likely to suffice, future studies should direct their attention towards a more integrative measurement, incorporating overall molecular biomarkers, physiological functional parameters as well as brain aging markers such as the one that we propose, the EEG SWA [125]. Longevity and healthy aging are not apparently synonymous but they should dovetail with each other. Taking into account the biomarkers that predict biological and brain aging, interventions promoting health even in advanced age can be indicated so that senescent changes and diseases' onset can be postponed or reversed and a life including fitness, vitality and time free of morbidity can be established [149]. In conclusion, although the discovery of the fountain of youth remains elusive, through the present thesis, specific lifestyles are recommended for successful aging and enhanced general, brain, body and sleep health.

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Appendices

Appendix A

A.1 Summary

By approaching the age of ninety, we will have spent approximately thirty years of our time sleeping; considering that, sleep must play a physiologically vital role in life irrespective of age. Although markedly puzzling and of enigmatic nature, both sleep and aging have been the subject of study for centuries, since almost all animals on the planet experience them. Several theories revolve around their existence and evolution converging on one point: along with several physiological processes that deteriorate in the course of aging, sleep is largely affected. In particular, stemming possibly from interrupted brain pathways and brain atrophy, sleep quality is considered to be progressively decreased in humans, due to attenuated capacity to initiate and/or maintain sleep. Sleep in the elderly includes decreased total sleep time and sleep efficiency, increased sleep fragmentation accompanied by problems falling asleep, and attenuated deeper sleep. Deeper sleep is reflected in the slow-wave activity (SWA) in the non-rapid eye movement (NREM) sleep electroencephalogram (EEG power in NREM sleep in 0.5-4 Hz), which in the elderly is found to be lower. In addition to sleep, the circadian clock, located in the suprachiasmatic nucleus of the hypothalamus, which normally controls rhythms in behavior and physiology with a period length of almost 24h, is also influenced by aging, where a reduction in the amplitude of its signal has been noted. A plethora of environmental factors, to which we are daily exposed, influence the sleep and circadian behavior from adulthood to aging. Since aging is an unavoidable process, an utmost goal of every individual is to achieve healthy aging with an augmented general body and brain health. The current thesis aims to investigate the way sleep and subsequently general health can be enhanced in the course of aging, exploring the detrimental and beneficial aspects of specific environmental factors, namely *diet, physical activity, light levels and caffeine intake*.

In order to establish the basis of how aging shapes the sleep architecture and sleep EEG in mice, we conducted an extensive study depicted in **Chapter 5**. Older mice were found to sleep more during the dark period compared to young ones, and antithetically to what it is shown in humans, SWA levels in NREM sleep were elevated. This study was particularly focused on the attributes of EEG slow waves, that were characterized by increased amplitude, steeper slopes and fewer multipeak waves. Our study suggests first, that aged mice are living under high sleep pressure conditions and, second, that altered brain network properties possibly prevail in aging.

Albeit the notable findings in **Chapter 5**, the mechanisms underlying these age-associated sleep changes remain elusive. Due to the complexity of the aging process, the development of a concrete and solid animal model is essential; however, in the sleep research field, in general, aging has not been profoundly studied since it comprises a strenuous and time consuming area. A need, therefore, has been developed for successful shorter timespan studies. Owing to that, in **Chapter 9**, we aimed to investigate sleep and the sleep EEG in a premature aging mouse model with a complete deficiency of the xeroderma pigmentosum group G protein (XPG). Animals with complete XPG deficiency usually have a very short life span (with a maximum life expectancy of 18 weeks) and display segmental progeria. Sleep architectural characteristics were found to be analogous to normally aged mice. Nevertheless, the sleep quality and brain integrity was likely compromised in this mutant model beyond normal aging, revealing an exacerbated aging condition usually not seen in naturally aged mice. The data point towards a different

sleep regulation in XPG deficient mice that awaits to be further studied.
One of the environmental factors directly linked with general health and longevity is diet. **Chapters 2 and 7** are dedicated in studying the effect of diet-induced obesity on sleep and its regulation in young adult and aged mice. In order to simulate the human condition, mice (6 and 18 months old) were fed for twelve weeks exclusively with high-caloric diet. Following chronic high-caloric diet, sleep architecture was moderately affected in young mice, including an increase in REM sleep during the light period, while regarding alterations in the EEG, lower NREM sleep SWA was found compared to controls. By applying mathematical modeling, an altered sleep homeostasis was demonstrated to be induced by high-caloric diet, establishing a bi-directional relationship between obesity and sleep. In contrast to young as discussed in **Chapter 2**, in aged mice **Chapter 7**, high-caloric diet had a significant impact on sleep architecture and sleep EEG. Particularly, more NREM sleep and less waking was found in both light and dark periods in high-caloric diet mice compared to age-matched controls, as well as an additional increase in the slow component of EEG SWA (0.5-2.5 Hz). Although aging is the dominant variable, the data denote a synergistic effect of aging and diet leading to the notion that chronically consumed high-caloric diet accentuates age-induced sleep changes.
A considerable factor, introduced in the previous century, is artificial light; albeit significant, it has been associated with drawbacks in health especially in humans when exposed during darkness. Dim-light-at-night, and its effects on sleep and the circadian clock in young and aged mice (18 and 24 months old) is the subject of discussion of **Chapters 4 and 8**. In **Chapter 4**, young mice were exposed to different duration periods of dim-light-at-night ranging from one day to three months. Sleep was already affected following merely one night of dim-light-at-night exposure, including a delay of vigilance state rhythms that increased as a function of dim-light-at-night exposure from one day to one month. Remarkably pronounced effects on sleep, rest-activity rhythms and the sleep EEG were noted following three continuous months of dim-light-at-night exposure, simulating data obtained from aged mice (**Chapter 8**), showing overall a sleep network deterioration. By conducting, in addition, a more elaborate mathematical analysis on the locomotor activity data, a less healthy rest-activity pattern was revealed in the long-term condition in young mice. In contrast to the young animals, EEG SWA in aged mice was differentially affected by long-term dim-light-at-night, as described in **Chapter 8**. Although a decrease was found in young mice, an increase in EEG SWA was shown in 24 months old mice while no alteration in the 18 months old compared to age-matched controls. Our results indicate that behavioral rhythmicity, sleep and brain integrity are compromised after chronic dim-light-at-night exposure in both young and age mice alike.
In contrast to factors that impair sleep and impede subsequently general health in the course of aging, other agents may cause opposite effects. An agent, generally considered to be beneficial for health across all ages is physical activity, which is studied in **Chapter 6**. Young (6 months old) and aged mice (18 and 24 months old) were provided with a running wheel in their cages for voluntary use on a daily basis for one to three months. The wheels were removed two weeks prior to the sleep recordings, therefore, a sustained effect of exercise was investigated. Sleep architecture was significantly affected by long-term exercise with the findings including an increased wakefulness during the dark period in young mice and mild alterations in the aged ones. Notably, long-term

exercise significantly counteracted the age-induced increase in SWA levels found in aging, as described in **Chapter 5**. Elaborating, it was found that long-term physical activity diminished the age-associated EEG SWA in levels very similar to young sedentary mice. By additionally performing machine learning approaches, we found that characteristic information regarding age and exercise was enclosed in the SWA data. Our data show that introduction of voluntary, long-term, age-matched exercise even later in life is able to bring about alterations towards a 'younger brain phenotype'. Through this study, we suggest that EEG SWA can be used as a biomarker of brain age and that physical activity could potentially be prescribed as a first-order 'medication' for body and brain health, throughout the whole age spectrum.

Another environmental factor, widely used in a daily basis, is caffeine whose intake effects on sleep behavior and the sleep EEG were investigated in young mice as described in **Chapter 3**. As a psychostimulant substance, when acutely consumed (i.e. one day) in the drinking water, it induced an increase in waking during the active phase and less deep sleep in the following rest phase, validating the common conjecture. In contrast, when caffeine in the drinking water was chronically consumed (for two weeks continuously), an increase in waking during the active period was found accompanied by an increase in sleep in the rest period. Additionally, EEG SWA in NREM sleep was markedly increased in the chronic condition compared to both the acute and control ones. The data suggest that, opposing the traditional conception on the impact on sleep, habitual caffeine consumption has effects on sleep and sleep regulation potentially providing a therapeutic window.

The current thesis investigates the effects of environmental inputs on sleep, circadian behavior and the sleep EEG in young and aged mice. Our data show that, effects are especially pronounced in young mice, denoting an increased environmental susceptibility, while, albeit present, they are more moderate in older mice. According to the results narrated in the present thesis, EEG SWA can be considered as a biomarker of brain age, marking well-defined aging signatures that reflect deleterious or advantageous effects on body and brain health owing to specific lifestyle choices. Concluding, although the elixir of youth has not been discovered yet, our data provide significant insights into environmental agents that intervene in the aging process and may allow for a healthier and longer life span.

A.2 Samenvatting

Als we de leeftijd van negentig jaar naderen, zullen we ongeveer dertig jaar van onze tijd hebben geslapen; gezien dat, slaap moet een fysiologisch vitale rol in het leven spelen, ongeacht de leeftijd. Hoewel opvallend raadselachtig van aard, zijn zowel slaap als veroudering eeuwenlang het onderwerp van studie geweest, aangezien bijna alle dieren op de planeet ze ervaren. Verschillende theorieën zijn ontwikkeld rondom hun bestaan en evolutie, die op één punt samenkomen: samen met verschillende fysiologische processen die verslechteren in de loop van veroudering, wordt de slaap grotendeels beïnvloed. Met name als gevolg van mogelijk onderbroken hersenpaden en hersenatrofie, wordt aangenomen dat de slaapkwaliteit bij mensen geleidelijk afneemt vanwege het verzwakte vermogen om de slaap te initiëren en / of in stand te houden. Slaap bij ouderen omvat verminderde totale slaaptijd en slaapefficiëntie, verhoogde slaapfragmentatie vergezeld van problemen bij het inslapen en verzwakte diepere slaap. Dieper slapen wordt weerspiegeld in de slow-wave-activiteit (SWA) in de non-rapid oogbewegingen (NREM) slaap slaap-elektro-encefalogram (EEG-vermogen in NREM-slaap in 0,5-4 Hz), die bij ouderen lager blijkt te zijn. Naast slaap de circadiane klok, gelegen in de suprachiasmatische kern van de hypothalamus, die normaal ritmen in gedrag en fysiologie regelt met een periode van bijna 24 uur, wordt ook beïnvloed door veroudering, waar een vermindering van de amplitude van het signaal werd opgemerkt. Een overvloed aan omgevingsfactoren, waaraan we dagelijks worden blootgesteld, beïnvloeden de slaap en het circadiane gedrag van volwassenheid tot veroudering. Aangezien veroudering een onvermijdelijk proces is, is het hoogste doel van elk individu om het verouderingsproces te ervaren met een verbeterde mentale en lichamelijke gezondheid. Het huidige proefschrift heeft tot doel de manier te onderzoeken waarop slaap en vervolgens de algemene gezondheid kunnen worden verbeterd tijdens het ouder worden, waarbij de schadelijke en heilzame aspecten van specifieke omgevingsfactoren worden onderzocht, namelijk *voeding, fysieke activiteit, lichtniveaus en inname van cafeïne*.

Om de basis te leggen voor hoe veroudering de slaaparchitectuur en het EEG bij muizen vormt, voerden we een uitgebreide studie uit, zoals beschreven in **Hoofdstuk 5**. Oudere muizen bleken meer te slapen in de donkere periode dan jonge, en in tegenstelling tot wat bij mensen wordt aangetoond, waren de SWA-waarden in NREM-slaap verhoogd. Deze studie was vooral gericht op de kenmerken van EEG-slow waves, die werden gekarakteriseerd door een grotere amplitude, steilere hellingen en minder multipeakgolven. Onze studie suggereert ten eerste dat bejaarde muizen onder hoge slaapdrukomstandigheden leven en, ten tweede, dat veranderde hersennetwerk eigenschappen mogelijk domineren bij veroudering.

Ondanks de opmerkelijke bevindingen in **Hoofdstuk 5**, blijven de mechanismen die ten grondslag liggen aan deze leeftijdsgebonden slaapveranderingen ongrijpbaar. Vanwege de complexiteit van het verouderingsproces is de ontwikkeling van een concreet en solide diermodel essentieel; op het gebied van slaaponderzoek is veroudering in het algemeen echter niet grondig bestudeerd, aangezien het een tijdrovend gebied is. Er is daarom behoefte ontstaan aan succesvolle kortere onderzoeken. Daarom wilden we in **Hoofdstuk 9** de slaap en het slaap-EEG onderzoeken in een vroegtijdig ouder wordend muismodel met een volledige deficiëntie van het xeroderma pigmentosum-groep G-eiwit (XPG). Dieren met volledige XPG-deficiëntie hebben meestal een zeer korte levensduur (met een maxi-

male levensverwachting van 18 weken) en vertonen segmentale progeria. Slaaparchitectuur kenmerken bleken analoog aan normaal verouderde muizen. Desalniettemin waren de slaapkwaliteit en de hersenintegriteit waarschijnlijk aangetast in dit mutante model, wat een verergerde verouderingstoestand aan het licht bracht die gewoonlijk niet wordt gezien bij natuurlijk verouderde muizen. De gegevens wijzen op een andere slaapregulatie bij muizen met XPG-deficiëntie die wachten op nader onderzoek.
Een van de omgevingsfactoren die rechtstreeks verband houden met de algemene gezondheid en levensduur is voeding. **Hoofdstukken 2 en 7** zijn gewijd aan het bestuderen van het effect van dieet-geïnduceerde obesitas op de slaap en de regulatie ervan bij jongvolwassen en oudere muizen. Om de menselijke conditie te simuleren, werden muizen (6 en 18 maanden oud) gedurende twaalf weken uitsluitend gevoerd met een calorierijk dieet. Na een chronisch hoogcalorisch dieet werd de slaaparchitectuur matig beïnvloed bij jonge muizen, waaronder een toename van de REM-slaap tijdens de lichtperiode, terwijl met betrekking tot veranderingen in de EEG, lagere NREM-slaap-SWA werd gevonden in vergelijking met controles. Door het toepassen van wiskundige modellering, werd aangetoond dat een hoogcalorisch dieet tot een veranderde slaaphomeostase leidt, wat een bi directionele relatie tussen obesitas en slaap tot stand bracht. In tegenstelling tot jonge muizen, zoals besproken in **Hoofdstuk 2**, had een hoogcalorisch dieet bij oudere muizen, **Hoofdstuk 7**, een significante invloed op de slaaparchitectuur en het slaap-EEG. In het bijzonder werd meer NREM-slaap en minder ontwaken gevonden in zowel lichte als donkere perioden bij muizen met een hoog calorisch dieet in vergelijking met controles van gelijke leeftijd, evenals een extra toename van de langzame component van EEG SWA (0,5-2,5 Hz). Hoewel veroudering de dominante variabele is, duiden de gegevens op een synergetisch effect van veroudering en voeding, wat leidt tot het idee dat chronisch geconsumeerd hoogcalorisch dieet leeftijd-geïnduceerde slaapveranderingen accentueert.
Een belangrijke factor die in de vorige eeuw werd geïntroduceerd, is kunstlicht; hoewel significant, is het in verband gebracht met nadelen voor de mensgezondheid, vooral bij blootstelling tijdens de nachtperiode. Dim-light-at-night en de effecten ervan op slaap en de circadiane klok bij jonge en oudere muizen (18 en 24 maanden oud) is het onderwerp van **Hoofdstukken 4 en 8**. In **Hoofdstuk 4** werden jonge muizen blootgesteld aan dim-light-at-night variërend van één dag tot drie maanden. De slaap werd al beïnvloed na slechts één nacht van blootstelling aan dim-light-at-night, inclusief een vertraging van de waakzaamheid ritmes die toenamen als functie van blootstelling aan dim-light-at-night van één dag tot één maand. Opvallende effecten op de slaap, rustactiviteitritmes en het slaap-EEG werden geconstateerd na drie aaneengesloten maanden van dim-light-at-night, waarbij gegevens van oudere muizen werden gesimuleerd (**Hoofdstuk 8**), die in het algemeen een verslechtering van het slaapnetwerk aantoonden. Door een meer uitgebreide wiskundige analyse uit te voeren op de bewegingsactiviteitgegevens, werd een minder gezond rustactiviteitspatroon onthuld in de langetermijn conditie bij jonge muizen. In tegenstelling tot jonge muizen gegevens werd EEG SWA bij oudere muizen differentieel beïnvloed door langetermijn dim-light-at-night, zoals beschreven in **Hoofdstuk 8**. Hoewel er een afname werd gevonden bij jonge muizen, werd een toename van EEG SWA binnen 24 maanden aangetoond bij oude muizen terwijl er geen verandering was in de 18 maanden oud vergeleken met controles van gelijke leeftijd. Onze resultaten geven aan dat gedragsritmiek, slaap en hersenintegriteit in gevaar zijn na chro-

nische blootstelling aan dim-light-at-night bij zowel jonge als oudere muizen.
In tegenstelling tot factoren die de slaap verstoren en vervolgens de algemene gezondheid belemmeren bij het verouderingsproces, kunnen andere middelen tegengestelde effecten veroorzaken. Een middel, dat algemeen wordt beschouwd als heilzaam voor de gezondheid van alle leeftijden is lichaamsbeweging en het wordt bestudeerd in **Hoofdstuk 6**. Jonge (6 maanden oud) en oude muizen (18 en 24 maanden oud) kregen een loopwiel in hun kooien voor vrijwillig dagelijks gebruik gedurende één tot drie maanden. Het loopwiel werd twee weken vóór de slaapopnames verwijderd en er werd een aanhoudend effect van lichaamsbeweging onderzocht. Slaaparchitectuur werd significant beïnvloed door langdurige inspanning, met als bevindingen een verhoogde waakzaamheid tijdens de donkere periode bij jonge muizen en milde veranderingen bij oudere muizen. Met name langdurige inspanning heeft de leeftijd-geïnduceerde toename van SWA-niveaus die bij veroudering worden aangetroffen, aanzienlijk tegengegaan, zoals beschreven in **Hoofdstuk 5**. Verder werd vastgesteld dat langdurige fysieke activiteit de leeftijd-gelinkt EEG-SWA verminderde in niveaus die sterk lijken op die van jongeren sedentaire muizen. Door daarnaast machine learning-benaderingen uit te voeren, ontdekten we dat kenmerkende informatie over leeftijd en lichaamsbeweging was opgenomen in de SWA gegevens. Onze gegevens tonen aan dat de introductie van vrijwillige, langdurige lichaamsbeweging, zelfs later in het leven, veranderingen in de richting van een 'jonger hersenfenotype' kan veroorzaken. Door deze studie suggereren we dat EEG SWA kan worden gebruikt als een biomarker van de hersenleeftijd en dat fysieke activiteit mogelijk kan worden voorgeschreven als eerste-orde 'medicatie' voor mentale en fysieke gezondheid, over het hele leeftijdsspectrum.
Een andere omgevingsfactor die dagelijks op grote schaal wordt gebruikt, is cafeïne waarvan de inname-effecten op slaapgedrag en het slaap-EEG werden onderzocht bij jonge muizen, zoals beschreven in **Hoofdstuk 3**. Als psychostimulerende stof, bij acute consumptie (dwz één dag) in het drinkwater, veroorzaakte cafeïne een toename van het ontwaken tijdens de actieve fase en minder diepe slaap in de rustfase, wat het algemene vermoeden bevestigde. Daarentegen, bij chronische cafeïne consumptie in het drinkwater (gedurende twee weken continu), werd een toename van het ontwaken tijdens de actieve periode gevonden, vergezeld van een toename van de slaap in de rustperiode. Bovendien was EEG SWA in NREM-slaap duidelijk verhoogd door chronische consumptie in vergelijking met zowel de acute als de controlegroep. De gegevens suggereren dat, in tegenstelling tot de traditionele opvatting over de impact op slaap, de gebruikelijke consumptie van cafeïne effecten heeft op de slaap en slaapregulatie, wat mogelijk een therapeutisch venster biedt.
Het huidige proefschrift gaat in op de effecten van omgevingsfactoren op slaap, circadiaan gedrag en slaap-EEG bij jonge en oudere muizen. Onze gegevens tonen aan dat de effecten vooral uitgesproken zijn bij jonge muizen, wat duidt op een verhoogde gevoeligheid voor omgevingsfactoren, terwijl ze, hoewel aanwezig, gematigder zijn bij oudere muizen. Volgens de resultaten in dit proefschrift, kan EEG SWA worden beschouwd als een biomarker van de hersenleeftijd. Tot slot, hoewel het elixer van de jeugd nog niet is ontdekt, bieden onze gegevens belangrijke inzichten in omgevingsfactoren die ingrijpen in het verouderingsproces en mogelijk een gezondere en langere levensduur mogelijk maken.

Α΄.3 Περίληψη

Όταν ένας άνθρωπος έχει φτάσει στα ενενήντα του χρόνια, θα έχει ξοδέψει περίπου τριάντα χρόνια από τη ζωή του στον ύπνο. Αναλογιζόμενοι αυτό, ο ύπνος πρέπει να παίζει ένα ζωτικής σημασίας ρόλο ανεξαρτήτου ηλικίας. Παρά την εντυπωσιακά αινιγματική τους φύση, ο ύπνος καθώς και η γήρανση έχουν αποτελέσει θέματα μελέτης για αιώνες, δεδομένου ότι σχεδόν όλα τα ζώα στον πλανήτη κοιμούνται και γερνούν. Πολλές θεωρίες έχουν προταθεί για την επεξήγηση της ύπαρξης και της εξέλιξης τους, οι οποίες συγκλίνουν σε ένα σημείο: όλες οι φυσιολογικές διαδικασίες υφίστανται επιδείνωση καθώς γερνάμε, έτσι και ο ύπνος. Ειδικότερα, προερχόμενη πιθανότατα από εγκεφαλική ατροφία ή κάποια ελαττωματική οδό στον εγκέφαλο, η ποιότητα του ύπνου στον άνθρωπο μειώνεται σταδιακά, εξαίτίας της μειωμένης ικανότητας του να ξεκινήσει τη διαδικασία του ύπνου και/ή να διατηρήσει τα επίπεδα του ύπνου. Στους ηλικιωμένους ο ύπνος συμπεριλαμβάνει μειωμένο συνολικό χρόνο και αποδοτικότητα, με αρκετές διακοπές που συνοδεύονται από θέματα που τους παρεμποδίζουν να αποκοιμηθούν με ευκολία, με περιορισμένο βαθύ ύπνο. Ο βαθύς ύπνος αντικατοπτρίζεται στα αργά και μακρά εγκεφαλικά κύματα που παρατηρούνται στο ηλεκτροεγκεφαλογράφημα (ΗΕΓ), επονομαζόμενα και κυματα Δέλτα (Δέλτα κύματα στο ΗΕΓ του ύπνου, 0.5 - 4 **Hz**), τα οποία στους ηλικιωμένους έχουν βρεθεί να εμφανίζονται με χαμηλότερη ηλεκτρική δραστηριότητα. Εκτός από τον ύπνο, και το κιρκαδικό ρολόι, το οποίο εντοπίζεται στον υπερχιασματικό πυρήνα του υποθαλάμου και ελέγχει τον συμπεριφορικό και φυσιολογικό ρυθμό με μια περίοδο που διαρκεί περίπου 24 ώρες, επηρεάζεται επίσης από τη γήρανση όπου έχει παρατηρηθεί μια μείωση του πλάτους του σήματος. Μία πληθώρα περιβαλλοντικών παραγόντων, στους οποίους είμαστε εκτεθειμένοι καθημερινά, επηρεάζουν την υπνική και κιρκαδική συμπεριφορά κατα τη διάρκεια της γήρανσης. Καθώς η γήρανση αποτελεί μια αναπόφευκτη διαδικασία, ο ύστατος σκοπός του κάθε ανθρώπου είναι να επιτύχει μια υγιή γήρανση με σαφή βελτίωση της σωματικής και εγκεφαλικής υγείας. Η παρούσα διατριβή στοχεύει στο να ερευνήσει τον τρόπο με τον οποίο ο ύπνος και κατ' επέκταση η γενικότερη υγεία μπορούν να βελτιωθούν κατα τη διάρκεια της διαδικασίας της γήρανσης, εξετάζοντας τις επιβλαβείς και ωφέλιμες διαστάσεις συγκεριμένων περιβαλλοντικών παραγόντων όπως, η διατροφή, η φυσική άσκηση, τα επίπεδα φωτος καθώς και η κατανάλωση καφεΐνης.

Θέτοντας τις βάσεις για να προσδιορίσουμε το πως η γήρανση διαμορφώνει την αρχιτεκτονική του ύπνου και το υπνικό ΗΕΓ στα ποντίκια, πραγματοποιήσαμε μια αναλυτική μελέτη που περιγραφεται στο Κεφάλαιο 5. Τα γηρασμένα ποντικια βρέθηκαν να κοιμούνται περισσοτερο κατά τη διάρκεια της νύχτας σε σύγκριση με τα νέα και, αντίθετα με τον άνθρωπο, τα επιπεδα των Δέλτα κυμάτων τους ήταν ψηλά. Αυτή η μελέτη, εστίασε στη εκτενή ανάλυση των κυμάτων αργής συχνότητας του ΗΕΓ τα οποία χαρακτηρίζονται από υψηλότερο πλάτος κύματος, πιο απότομες κλίσεις και με λιγότερες κορυφές. Τα δεδομένα υποδηλώνουν, κατ'αρχήν ότι τα γηρασμένα ποντικια ζουν κάτω από συνθήκες μεγάλης υπνικής πίεσης και κατά δεύτερον, ότι στη γήρανση οι ιδιότητες των εγκαφαλικλων δικτύουν αλλάζουν.

Παρά τα αξιοσημείωτα ευρήματα του Κεφαλαίου 5, οι μηχανισμοί που αποτελούν τη βάση των αλλαγών που παρατηρήθηκαν στον ύπνο δεν εχουν ακόμη αποσαφηνιστεί. Η γήρανση αποτελεί μία πολύπλοκη διαδικασία, και για αυτό το λόγο η ύπαρξη ενός πειραματικού μοντέλου είναι απαραίτητη. Παρ'όλα αυτά, η γήρανση δεν εχει μελετηθεί

σε βάθος στον τομέα του ύπνου, καθώς είναι ένα χρονοβόρο και επίπονο επιστημονικό πεδίο. Έχει δημιουργηθεί συνεπώς η ανάγκη για τη δημιουργία επιτυχημένων αλλά μικρότερης χρονικής διάρκειας ερευνών. Για αυτό το λόγο, στόχος του Κεφαλαίου 9 ήταν η μελέτη των πειραματόζωων προγηρίας που είχαν έλλειψη του συμπλέγματος πρωτεινών της Μελαγχρωματικής ξηροδερμίας. Τα πειραματόζωα με παντελή έλλειψη του συμπλέγματος έχουν συνήθως μικρή διαρκεια ζωής (το μέγιστο προσδόκιμο ζωής τους είναι 18 εβδομάδες) και εμφανίζουν τμηματική προγηρία. Η αρχιτεκτονική του ύπνου βρέθηκε να είναι παρόμοια με αυτή των φυσιολογικά γηρασμένων ποντικιών. Ωστόσο, η ποιότητα του ύπνου και η εγκεφαλική ακεραιότητα είχε μειωθεί στα πειραματόζωα προγηρίας πέρα από τα φυσιολογικά επίπεδα γήρανσης, φανερώνοντας μία τρομερή επιδείνωση της γήρανσης. Τα αποτελέσματα δείχνουν οτι πιθανόν η λειτουργία του ύπνου να ελέγχεται διαφορετικά στα πειραματόζωα προγηρίας που μπορεί στο μέλλον να μελετηθεί εκτενέστερα.

Ένας από τους περιβαλλοντικούς παράγοντες άμεσα συνδεδεμένος με την υγεία και τη μακροβιότητα ειναι η διατροφή. Τα Κεφάλαια 2 και 7 μελετούν την επίδραση της παχυσαρκίας στον ύπνο και τη λειτουργία του σε νέα και γηρασμένα ποντίκια. Για να προσομειώσουμε τις συνθήκες που επικρατούν στον άνθρωπο, τα ποντίκια (6 και 18 μηνών) τρέφονταν αποκλειστικά για δώδεκα εβδομάδες με τροφή μεγάλης θερμιδικής αξίας. Μετά από αυτήν την μεγάλης διάρκειας αλλαγή στη διατροφή, ο ύπνος επηρεάστηκε σε μέτριο βαθμό στα νεαρής ηλικίας ποντίκια δείχνοντας μια αύξηση στον ύπνο γρήγορων κινήσεων των ματιών (rapid-eye-movement sleep, REM) κατα τη διάρκεια της μέρας και χαμηλότερα επιπεδα Δέλτα κυμάτων. Με την εφαρμογή ενός μαθηματικού μοντέλου, βρήκαμε μια αλλαγή στην ομοιόσταση του ύπνου μετά απο διατροφή μεγάλης θερμιδικής αξίας, γεγονός που εδραιώνει την αμφίδρομη σχέση μεταξύ παχυσαρκίας και ύπνου. Σε αντίθεση με τα νέα ποντίκια, όπως εξετάζεται στο Κεφάλαιο 2, τα γηρασμένα ποντίκια που μελετώνται στο Κεφάλαιο 7 επηρεάστηκαν σφόδρα από την αλλαγή στη διατροφή. Παρέμεναν λιγότερο ξύπνια και κοιμούνταν περισσότερο κατά τη διάρκεια και της μέρας και της νύχτας συγκριτικα με τα ίδιας ηλικίας ποντίκια που τρέφονταν κανονικά, και επιπροσθέτως, παρατηρήθηκε μια αύξηση στην ηλεκτρική δραστηριότητα της μικρότερης συχνότητας των επιπέδων των Δέλτα κυμάτων (0.5-2.5 Hz). Παρόλο που η γήρανση είναι η βασική παράμετρος, τα δεδομένα μας υποδεικνύουν ότι υπάρχει μια συνέργεια της γήρανσης και της διατροφής που οδηγεί στην ιδέα ότι η μακροχρόνια κατανάλωση τροφών υψηλής θερμιδικής αξίας ενισχύει τις αλλαγές στον ύπνο που παρατηρούνται μόνο από τη γήρανση.

Ένας σημαντικός παράγοντας, που συστήθηκε στην ανθρωπότητα τον προηγούμενο αιώνα είναι το τεχνητό φως. Παρά τη σημασία του, έχει συνδεθεί με προβλήματα στην υγεία ιδιαίτερα στη έκθεση μας κατά τη διάρκεια της νύχτας. Το απαλό φως τη νύχτα και οι επιδράσεις του στον ύπνο και τον κιρκαδικό ρολόι σε νέα και γηρασμένα ποντίκια (18 και 24 μηνών), αποτελεί το θέμα έρευνας των Κεφαλαίων 4 και 8. Στο Κεφάλαιο 4, τα νέα ποντίκια εκτέθηκαν σε περιόδους διαφορετικής διάρκειας νυχτερινού απαλού φωτός, σε ένα εύρος απο μία μέρα έως τρεις συνεχόμενους μήνες. Ο ύπνος άλλαξε ραγδαία από την πρώτη κιόλας νύχτα έκθεσης και συμπεριλάμβανε μια καθυστέρηση του ρυθμού του ύπνου η οποία αυξανόταν γραμμικά με την αύξηση της περιόδου έκθεσης στο απαλό φως. Μετά από τρεις συνεχόμενους μήνες έκθεσης στο απαλό φως, σημαντικές αλλαγές παρατηρήθηκαν στον ύπνο και το ρυθμό του στη διάρκεια της μέρας και στο ΗΕΓ, παρουσιάζοντας ομοιότητες με τα δεδομένα από τα

γηρασμένα ποντίκια Κεφάλαιο 8, δείχνοντας συνολικά μία επιδείνωση της συνοχής του ύπνου. Με την εφαρμογή μίας λεπτομερούς μαθηματικής ανάλυσης στα δεδομένα της δραστηριότητας των ποντικιών, βρέθηκε ένα όχι και τόσο θετικό προς την υγεία μοτίβο συγκεκριμένα όταν τα ποντίκια είχαν εκτεθεί σε μεγάλης χρονικής διάρκειας φως τη νύχτα. Σε αντίθεση με τα δεδομένα των νεαρών ποντικιών, τα Δέλτα κύματα στο ΗΕΓ επηρεάστηκαν αλλιώς στα γηρασμένα ποντίκια, όπως περιγράφεται στο Κεφάλιο 8. Μία μείωση της ηλεκτρικής δραστηριότητας τους είχε βρεθεί στα νέα ποντίκια, ενώ στα 24 μηνών ποντίκια μία αύξηση της ηλεκτρικής δραστηριότητας των Δέλτα κυμάτων ήταν εμφανής και καμία αλλαγή δεν βρέθηκε στα 18 μηνών ποντίκια, οπώς αυτα συγκρίθηκαν με συνομίληκα τους που δεν εκτέθηκαν σε απαλό φως τη νύχτα. Τα αποτελέσματα μας υποδηλώνουν ότι ο συμπεριφορικός ρυθμός, και η συνοχή των λειτουργιών του ύπνου και του εγκεφάλου υπέστησαν βλάβες μετά τη μακροχρόνια έκθεση των ποντικιών σε απαλό φως τη νύχτα κατά τον ίδιο τροπο στα νέα και γηρασμένα.

Αντίθετα με τους παράγοντες που είναι επιβλαβείς για τον ύπνο και κατ'επέκταση παρεμποδίζουν την υγιή γήρανση, υπάρχουν άλλοι που μπορούν να επιφέρουν οφέλη. Ένας παράγοντας που θεωρείται γενικότερα ωφέλιμος για την υγεία σε όλες τις ηλικίες είναι η φυσική άσκηση, η οποία μελετάται στο Κεφάλαιο 6. Νέα (6 μηνών) και γηρασμένα ποντίκια (18 και 24 μηνών) είχαν στα κλουβιά τους μία ρόδα άσκησης, την οποία μπορούσαν να χρησιμοποιήσουν όποτε αυτά ήθελαν σε καθημερινή βάση, για ένα διάστημα από ένα έως τρεις μήνες. Οι ρόδες αφαιρέθηκαν από τα κλουβιά των ποντικιών δύο βδομάδες πριν από την πειραματική καταγραφή του ύπνου, και ως εκ τούτου, μελετήσαμε τα παρατεταμένα αποτελέσματα της άσκησης. Ο ύπνος επηρεάστηκε σημαντικά από τη μακροχρόνια άσκηση με τα ευρήματα να δείχνουν ότι τα ποντίκια ήταν περισσότερη ώρα ξύπνια κατά τη διάρκεια της ενεργής περιόδου τους (νύχτα), ενώ ήπιες αλλαγές υπέστη ο ύπνος των γηρασμένων ποντικιών. Εξαιρετικό εύρημα αποτελεί η αλλαγή στα επίπεδα της ηλεκτρικής δραστηριότητας των Δέλτα κυμάτων, όπου διαφαίνεται κατά μία έννοια η καταστολή της γήρανσης σε αυτά, που αποτελούσε θέμα συζήτησης του Κεφαλαίου 5. Πιο αναλυτικά, βρήκαμε ότι η μακροχρόνια άσκηση μπορούσε να μειώσει τα επίπεδα της ηλεκτρικής δραστηριότητας των Δέλτα κυμάτων σε αντίστοιχα νεαρών ποντικιών που δεν είχαν αθληθεί ποτέ. Με μεθόδους μηχανικής μάθησης (machine learning), διαπιστώσαμε ότι χαρακτηριστικές πληροφορίες σχετικά με την ηλικία και τα επίπεδα άσκησης εμπεριέχονταν στα Δέλτα κύματα. Τα επιστημονικά δεδομένα μας δείχνουν ότι η εθελοντική, μακροχρόνια και κατάλληλη για την ηλικία άσκηση, ακόμη και αν αυτή εισαχθεί μεταγενέστερα στη ζωή ενός ανθρώπου, μπορεί να επιφέρει αλλαγές που υποδηλώνουν έναν 'νεότερο εγκεφαλικό φαινότυπο'. Μέσα από αυτή τη μελέτη, προτείνουμε οτι τα Δέλτα κύματα μπορούν να χρησιμοποιηθούν σαν βιοδείκτης (biomarker) της ηλικίας του εγκεφάλου καθώς και ότι η φυσική άσκηση θα μπορούσε να συνταγογραφηθεί σαν πρώτης επιλογής 'φαρμακευτική αγωγή' για τη σωματική και πνευματική υγεία, σε όλο το φάσμα ηλικιών.

Η καφεΐνη αποτελεί μια ουσία που καταναλώνεται ευρέως καθημερινά, οι επιδράσεις της οποίας στον ύπνο και το ΗΕΓ αποτελούν το θέμα μελέτης του Κεφαλαίου 3. Δρώντας σαν μία αναμενόμενη ψυχοδιεγερτική ουσία, όταν η καφεΐνη καταναλώθηκε άμεσα στο πόσιμο νερό των ποντικιών (μια μέρα), προκάλεσε εγρήγορση στα ποντίκια στη διάρκεια της ενεργής περιόδου τους (νύχτα), και μείωσε τον βαθύ ύπνο στην ακριβώς επόμενη ήρεμη περίοδο τους (μέρα). Αντίθετα, όταν τα ποντίκια μπορούσαν να κατα-

ναλώσουν καφεΐνη μέσα στο πόσιμο νερό τους για μεγαλύτερο χρονικό διάστημα (για δύο εβδομάδες συνεχόμενα) έντονη δραστηριότητα βρέθηκε στη διάρκεια της ενεργής περιόδου, την οποία ακολουθούσε μια σημαντική αύξηση του ύπνου στην ήρεμη περίοδο. Επιπροσθέτως, η ηλεκτρική δραστηριότητα των Δέλτα κυμάτων του ΗΕΓ κατά τη διάρκεια του ύπνου, αυξήθηκε σημαντικά στη χρόνια κατανάλωση καφεΐνης συγκριτικά με την άμεση και τη μη κατανάλωση. Τα επιστημονικά δεδομένα μας υποδεικνύουν ότι αντίθετα με την παραδοσιακή αντίληψη για τη δράση της καφεΐνης έναντι του ύπνου, η κατά συρροήν κατανάλωσή της επιφέρει αλλαγές στον ύπνο και τη λειτουργία του, προσφέροντας τη δυνατότητα να χρησιμοποιηθεί θεραπευτικά.
Η παρούσα διατριβή εμβαθύνει στις επιδράσεις των περιβαλλοντικών παραγόντων στον ύπνο, την κιρκαδική συμπεριφορα και το ΗΕΓ σε νέα και γηρασμένα ποντίκια. Τα επιστημονικά ευρήματά μας φανερώνουν ιδιαίτερα σοβαρή επίδραση των περιβαλλοντικών παραγόντων στα νεαρά ποντίκια, υποδηλώνοντας ότι αυτά διαθέτουν μία ευπάθεια, ενώ, παρ'ότι υπάρχει, η αντίστοιχη επίδραση στα γηρασμένα ποντίκια είναι γενικότερα πιο ήπια. Σύμφωνα με τα δεδομένα που αφηγήθηκαν στην παρούσα διατριβή, τα Δέλτα κύματα στο ΗΕΓ μπορούν να χρησιμοποιηθούν σαν βιοδείκτης της ηλικίας του εγκεφάλου, σηματοδοτώντας χαρακτηριστικά της γήρανσης που μαρτυρούν επιβλαβή ή ωφέλιμα αποτελέσματα στην σωματική και πνευματική υγεία εξ'αιτίας συγκεριμένων επιλογών διαβίωσης. Συνοψίζοντας, παρόλο που το ελιξήριο της νεότητος δεν έχει ανακαλυφθεί ακόμη, τα επιστημονικά ευρήματά μας συνθέτουν μία εικόνα στο πως οι περιβαλλοντικοί παράγοντες μπορούν να επέμβουν στη διαδικασία της γήρανσης ώστε να συμβάλουν στην υγεία και τη μακροβιότητα.

Appendix B

B.1 Acknowledgments

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B.2 Curriculum Vitae

Maria Panagiotou was born on the 7th of January 1988 in Athens, Greece. In 2005, she started her studies in the School of Pharmacy in the National and Kapodistrian University of Athens. During the last academic year in 2010, through the Erasmus program, she visited Rome where she stayed for a semester to conduct her research and produce her thesis in Neuroscience, in the Physiology lab in La Sapienza University. She completed her studies in Athens later that year, when she obtained her bachelor's and master's degree in Pharmacy. During the Erasmus program, she encountered the research in the field of Neuroscience, which she loved and wanted to explore further. Therefore, in 2011, she continued her studies in the Cognitive Neuroscience program in Radboud University of Nijmegen, the Netherlands. Following one year of multiple courses, and one year performing research in the Donders Institute, in a collaboration with the pharmaceutical company Chardon Pharma, under the supervision of Tineke van Rijn & Ard Peeters, she completed her research master's degree in 2013. In 2014, she began her phD studies in the Neurophysiology lab in Leiden University Medical Center in Leiden, the Netherlands in the group of Joke Meijer, under the supervision of Tom de Boer. Since 2019, she is appointed as lecturer in the University Medical Center Hamburg, located in the center of Hamburg, Germany, where she currently lives.

B.3 List of publications

- *Panagiotou M* & Deboer T. "Effects of chronic dim-light-at-night exposure on sleep in young and aged mice." Neuroscience 426 (2020): 154-167

- van Aken MA, Groothuis PG, *Panagiotou M*, Duin MV, Nap AW, van Rijn TC, Kozicz T, Braat DD, Peeters AB. "An objective and automated method for evaluating abdominal hyperalgesia in a rat model for endometriosis." Lab Anim (2019) 1:23677219856915 doi: 10.1177/0023677219856915

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